Transformation of microspore-derived embryos of winter oilseed rape (*Brassica napus* L.) using *Agrobacterium tumefaciens*

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Abstract

Microspore–derived embryos (MDEs) of *Brassica napus* present suitable materials for introduction of foreign genes. MDEs generally exhibit a high regeneration potential. Also, as a result of chromosome duplication, the introduced trait can be evaluated in a homozygote. The aim of this study was to develop a method for delivery of foreign genes into winter oilseed rape microspore-derived embryos using *Agrobacterium tumefaciens* strains. 21-day-old MDEs of homozygous line DH O120 were conditioned for transformation by exposure to low temperature for 2 weeks. Next, they were put for a few days in the growth room and then inoculated with *Agrobacterium*. The *A. tumefaciens* strains EHA105 and LBA4404 were used for transformation, both carrying the binary vector pKGIB containing the GUS gene with an intron under control of CaMV 35S RNA promoter and the *bar* gene under control of NOS promoter as a selection marker. Plants regenerated from transformed MDEs were resistant to herbicide Basta as it was confirmed by a test of leaf painting. The PCR analyses confirmed the presence of *bar* gene in the genomic DNA of obtained plants. All plants of T_0 generation were haploid before seed set. The chromosome doubling was conducted using the method of colchicine treatment of secondary axillary shoots. Plants of T_1 generation obtained from seeds collected from primary transformants were analysed for presence of the introduced *bar* gene. The progeny plants of the transformant DH T-39 were all transgenic as they inherited the transgene from double haploid, homozygous parent. T-DNA was stably transmitted into progeny plants as it was confirmed by Southern-blot hybridization of revealing the integration of T-DNA into winter oilseed rape genomic DNA.

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

Introduction

High frequency of plant regeneration is required for successful gene transfer and recovery of the transgenics. Microspore-derived embryos (MDEs) generally exhibit a high regeneration potential. No more than ten per cent of microspores, obtained from stimulating to androgenesis of microspores in isolated microspore culture, have gone spontaneous chromosomes duplication. Thus haploid microspore-derived embryos are potentially useful as recipients of foreign genes, especially if the frequency of microspore embryogenesis is high. Also, as a result of artificial chromosome duplication, the introduced gene can be evaluated in the homozygote. *Agrobacterium tumefaciens* –mediated transformation is in general a much more efficient way of introducing foreign genes than other techniques (Takahata et al., 2005). The breeding of transgenic plants is an extention of other breeding technologies and suggests introducing the new useful traits. Several cultivars and hybrids of oilseed rape with new traits were obtained as resistance to insects and herbicides or with a new composition of fatty acid (Zhong, 2001). Transgenic plants can be used for the basic study to explain metabolism, in e.g. glucosinolates in oilseed rape (Troczyńska et al. 2004). Therefore it is important to possess replicable and efficient methods to obtain the transgenic plants.

In the investigation reported below we developed a method for delivery of foreign genes into winter oilseed rape MDEs with the use of *Agrobacterium tumefaciens.* The PCR analysis of the next generation of transformants developed from the transformed microspore-derived embryos was performed as well.

Materials and methods

Plant material: The culture of microspore-derived embryos was carried out according to Cegielska et al. (2002) using winter oilseed rape (*Brassica napus* L.) line DH-O-120.

Bacterial strains and plasmid: We used two bacterial strains of *Agrobacterium tumefaciens* – LBA4404 and EHA 105, each carrying binary plasmid pKGIB, constructed for the experiments (Fig. 1). The plasmid contained the selection marker gene *bar*, encoding phosphinotricin (PPT) acetyltransferase (PAT), under the control of constitutive nopaline synthase (NOS) promoter and the reporter gene *uidA* encoding β-glucuronidase (GUS) with an intron, under the control of constitutive 35S RNA CaMV promoter. The plasmid was mobilized into strains of *Agrobacterium tumefaciens* EHA105 and LBA4404 by electroporation. The *Agrobacterium tumefaciens* suspension was prepared according to Pniewski and Kapusta (2005).

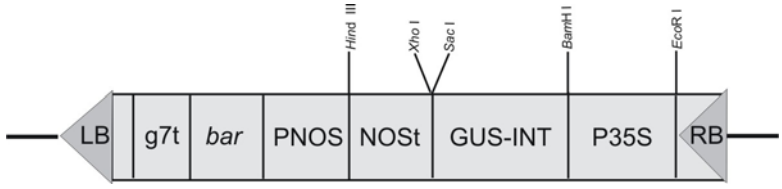

Fig. 1. Organisation of T-DNA of the binary plasmid pKGIB P35S = CaMV 35S promoter; PNOS = nopaline synthase promoter; GUS-INT = gene of β-glucuronidase with an intron; BAR = gene of phosphinotricin acetyltransferase; NOSt = nopaline synthase terminator; g7t = g7 terminator; LB and RB = left and right border sequences

Transformation of microspore-derived embryos: The 21–day-old microspore-derived embryos growing on B_5 (Gamborg,et al.1968) medium with 0.1 mg $l^{-1}GA_3$ were exposed to low temperature (1^0C) for two weeks. Next, they were put for a few days in the growing chamber. Thus prepared MDEs were used for transformation procedure. Cotyledons and hypocotyls were punctured with a needle and inoculated with *Agrobacterium tumefaciens* suspension for two hours. Then the explants were placed onto basic B_5 medium without antibiotic. The explants were cocultivated with *A. tumefaciens* for two days and then transferred to B_5 medium supplemented with an antibiotic (300 mg l^{-1} timentin) and phosphinotricin (10 mg l^{-1}) for four weeks.

Regeneration plants and doubling of chromosome number: After the period of culture on B_5 medium green explants were transferred to shoot induction medium MS (Murashige and Skoog 1962) containing 0.1 mM kinetin supplemented with timentin and phosphinotricin. The explants were transferred to fresh medium every two weeks. Green shoots were placed onto root induction medium (MS with 10mgl^{-1} IBA). Plantlets were put to soil. The chromosome doubling of haploids was made by the method of colchicine treatment of secondary axillary shoots. The putative transgenic plants developed under greenhouse conditions to the seed set.

PCR analysis of T0 and T_1 transformants: Genomic DNA from transgenic plants was isolated in a small-scale procedure according to the modified CTAB method (McGarvey and Kaper 1991). Polymerase chain reactions (PCR) were set up in a volume of 20 µl containing approximately 100 ng of plant genomic DNA, 0.2 mM of each dNTP, 10 pM of each primer and using 1 unit of *Taq* DNA polymerase (Biometra). The PCR temperature profile was set as follows: initial denaturation at 94ºC for 4 min, next 35 cycles of denaturation at 94ºC for 1 min, annealing for 45 sec, elongation at 72ºC for 1 min, and a final extension at 72ºC for 5 min. Primers and the *bar* gene were designed (primer F: 5′-CTG CAC CAT CGT CAA CCA CTA CAT C-3′; primer R: 5′-CTG AAG TCC AGC TGC CAG AAA CC-3′; primers annealed at 65ºC, amplified DNA fragment of 444 bp in length).

Additional screening of plant genomic DNA of T_0 transformants by the PCR analysis was done to exclude *Agrobacterium* DNA residual contamination. 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′; primers annealed at 62ºC) for the *Agrobacterium* chromosomal *chvA* gene were designed to amplify the 777 bp DNA fragment. The PCR reactions were set up as previously. All the PCR reactions were performed in the PTC-200 MJ Research thermal cycler. Amplification products were analysed by agarose gel electrophoresis. Molecular weight of products was estimated with the use of a 100 bp ladder (MBI Ferments) as a standard.

Southern blot analysis of transgenic plants: Genomic DNA from plants was isolated according to the modified CTAB method (Chee and Slightom 1994). 20–25 µg of DNA was digested with *Eco*R I and after electrophoresis blotted onto a positively charged nylon membrane (Roche). The probe was prepared by the PCR amplification of the *bar* gene with the use of a DIG labelling kit (Roche) and hybridized according to the manufacturer's protocol. CSPD was used as a chemiluminescent substrate and the signals were detected on an X-ray film.

GUS assay and Basta tests: The histochemical GUS assay was carried out on transformed embryos according to Jefferson (1987). Plants at four leaves stage were sprayed with the solution of herbicide Basta containing 0.1 % of Basta. Alternatively, leaflets were painted with the solution on the upper side.

Results and discussion

The use of androgenic forms to transformation, e.g. microspore-derived embryos, seems to be convenient and advantageous because from the beginning of the process it may possess biological material uniform and characterized with high ability to regeneration. Above all the progeny of the doubled haploid thus homozygous transgenic plant should be transgenic too.

MDEs were stimulated to growth by treatment of low temperature (1^0C) before inoculation of two strains of *A. tumefaciens* EHA 105 and LBA4404. Histochemical GUS assay in MDEs conducted ten days after inoculation confirmed the expression of introduced genes at the stage of the beginning of plant regeneration (Fig. 2). The plants developed from transformed MDEs, regenerated on a medium with 10 mgl^{-1} PPT, were transferred into the soil. Plants at four leaves stage were tested for the resistance to herbicide Basta containing PPT. Among the seventeen plants regenerated from transformed embryos of DH –O120 line five plants were completely resistant to herbicide. In the next step all regenerated plants were studied considering the presence of *bar* gene in genomic DNA by the use of PCR method. Transgene was detected in nine putative transformants. The results were verified using the test for presence of the residual *A. tumefaciens* cells in plant tissue. Integration of T-DNA containing *bar* gene was confirmed by Southern hybridization analysis. One copy of T-DNA was detected in part of plants but in plant DH T-39 two places of integration were detected (Fig.3). After the doubling of

chromosome of obtained transgenic haploids the fertile plants were separately isolated and grown to seed set. Inheritance of the transgene in the T_1 generation was studied for twenty progeny plants of all nine T_0 transformants PCR (+). All progeny plants of the doubled haploid DH T-39 revealed presence of the *bar* gene (Fig. 4). The result indicates that transgenic haploid plants after chromosome doubling result in homozygous T_1 plants in 100% transgenic.

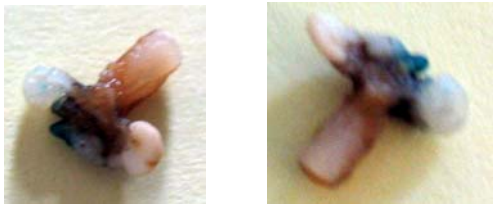

Fig. 2. GUS expression in microspore-derived embryo several days after transformation using *A. tumefaciens*

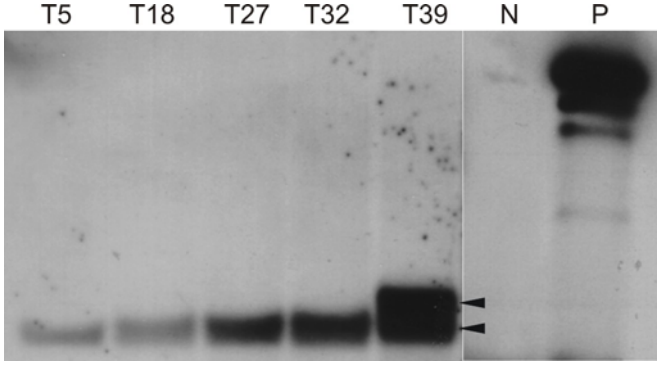

Fig. 3. Southern-blot analysis of transgenic plants of oilseed rape DH line O-120. Lanes: T-5 – T-39 = transgenic plants of oilseed rape DH line O-120; N = non-transgenic plant, negative control; P – plasmid pKGIB digested with *Eco*R I, positive control

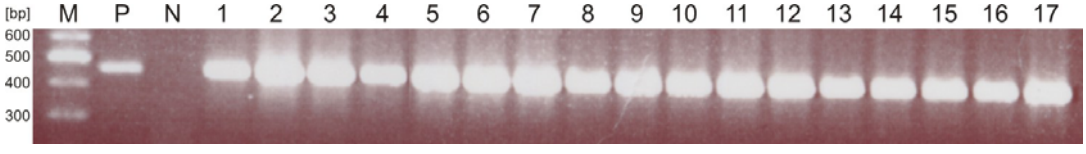

Fig. 4. PCR analysis of the *bar* gene in T_1 plants of DH T-39 transformant of oilseed rape DH line O-120.

Lanes: M = DNA size marker; P = plasmid pKGIB, positive control; N = non-transformed O-120 plant, negative control; 1-17 = T_1 plants of DH T-39 transformant

Conclusion

The presented method permitted to obtain stable transgenic DH line of winter oilseed rape.

Our further study will be concentrated on optimization of the transformation efficiency and then introduction of useful genes for agriculture traits.

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