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J. L. Chen · W. D. Beversdorf

A combined use of microprojectile bombardment and DNA imbibition enhances transformation frequency of canola (*Brassica napus* L.)

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Abstract Efforts to increase the frequency of recovered homozygous transgenic *B. napus* plants from direct DNA transformation treatments led to the development of a method of combined microprojectile bombardment and desiccation/DNA imbibition. The combined method was compared to individual treatments in two experiments utilizing microspore-derived embryo hypocotyls as targets for the β-glucuronidase (GUS) and NPT II genes. Both the transient gene expression of β-GUS and the stable transformation by NPT II demonstrated that the combined use of microprojectile bombardment and desiccation/DNA imbibition yielded more transgenic plants (at least three-times more) than either individual transformation protocol. In a histochemical analysis for β-GUS activity, an average of 37% of the hypocotyls receiving the combined treatment displayed a positive response, whereas only 8% of the hypocotyls showed a positive response following microprojectile bombardment alone. The hypocotyls obtained by the joint treatment also showed more multisite expression of the β-GUS gene per hypocotyl than those treated only with microprojectile bombardment. Southern analysis of NPT II gene integration into subsequently-derived secondary embryos indicated that the transformation efficiency of the combined treatment was 2% in comparison to 0.6% for that of the singular microprojectile bombardment. The number of inserts integrating per transformation event appears to be independent of the transformation methods. Neither of the marker genes was expressed in hypocotyls treated only with desiccation/DNA imbibition. Utilization of hypocotyl regeneration from microspore-derived embryos via a secondary embryogenesis system provided a reliable method for producing transgenic plants. The combined use of microprojectile bombardment and desiccation/DNA imbibition proved to be an efficient approach to obtain homozygous transgenic canola plants.

Key words Canola · Desiccation/DNA imbibition · Microprojectile bombardment · Microspore-derived embryos · Transformation

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J. L. Chen[1] (✉) · W. D. Beversdorf[2]
Department of Crop Science, University of Guelph, Ontario N1G 2W1, Canada

Present addresses:
[1] Biotech lab, Plant Genetic Systems (Canada) Inc. #104-111 Research Drive, Saskatoon, Saskatchewan S7N 3R2, Canada
[2] Ciba Seeds, P.O. Box 18300, Greensboro, NC 27419-8300, USA

Introduction

Plant transformation systems have opened new avenues for gene transfer between species in a rapid and specific manner. Numerous transformation approaches have been attempted in different species. *Agrobacterium*-mediated transformation, although in some cases used in monocotyledonous species, is an efficient system primarily for dicotyledonous species (Gasser and Fraley 1989). Microprojectile bombardment, first developed by Klein et al. (1987), has been used extensively to introduce foreign genes into both dicotyledonous and monocotyledonous species. Although host limitation is largely eliminated by microprojectile bombardment, one technical problem with this procedure is the unpredictable frequency of transient and integrative events (Potrykus 1991). Direct DNA uptake has caught the interest of scientists because of its simplicity. Transgenic plants were produced in this way using protoplasts, by either introducing DNA by electroporation (Shillito et al. 1985) or by simple chemical treatment of the cells (Negrutiu et al. 1987), or else through silicone-carbide treatment of plant cells (Kaeppler et al. 1992). However, reliability and efficiency are still the two major problems associated with these methods. A very simple, perhaps primitive sounding method for DNA uptake involves desiccation and plant tissue imbibition in a DNA-containing solution (Potrykus 1991). This approach is not a new one having been tried since the early days of the

development of plant transformation systems. Since these early but controversial results of DNA uptake by dry plant tissue using imbibition (Hess 1969; Ledoux and Huart 1969; Kleinhofs et al. 1975; Kleinhofs and Behki 1977), there has been a continuous effort to make it more reliable (Soyfer 1980; Topfer et al. 1989; Chen 1991; Senaratna et al. 1991). The extremely-low efficiency of DNA uptake by desiccation/DNA imbibition may reflect limited DNA penetration of the cell wall and membranes.

In order to increase transformation efficiency using a microprojectile-bombardment-based system, we explored a joint approach: the combined use of microprojectile bombardment and desiccation/DNA imbibition. In conjunction with this transformation system, we also used a reliable tissue culture method, involving secondary embryos obtained from microspore-derived (haploid) embryos of oilseed rape (*Brassica napus*). Using these transformation and tissue culture systems, we report here an increased frequency (at least three-times that of either separate treatment) of stably-transformed homozygous plants containing marker transgenes.

Materials and methods

Experiments

Two experiments, each involving three transformation treatments applied to 300 microspore-derived embryos (targets) per protocol, were conducted. Both experiments employed transformation protocols of microprojectile bombardment, desiccation/DNA imbibition and the combined treatment.

The first experiment involved the β-glucuronidase (β-GUS) gene present in pBI221.2 (kindly provided by Dr. L. Erickson, University of Guelph). In this experiment, transformation treatments were evaluated by histochemical analysis of the transformation targets.

The second experiment employed kanamycin resistance associated with plasmid pA11 NPTII (kindly provided by the former Allelix Inc, Mississauga, Ontario). Transformation treatments in this experiment were evaluated following recovery of secondary embryo-derived plants which survived culture with kanamycin at 100 mg/l by using both PCR and Southern analyses.

Microspore-derived targets

Microspore-derived embryos of *B. napus* L. line M3–124 were produced by the procedure of Coventry and Kott (1988). Microspores isolated from the donor plants were resuspended at a density of 80 000 microspores/ml, in Lichter's liquid medium (Lichter 1982) supplemented with 13% sucrose in 100 × 15 mm Petri dishes, each containing 10 ml of the liquid medium. The plates were incubated in darkness at 30 °C for 2 weeks, when globular embryos became visible; they were diluted to approximately 20 to 40 embryos per 10 ml of the same culture medium and allowed to further develop in a shaker (60 rpm) until most embryos had formed two large cotyledons (Coventry and Kott 1988). Cotyledons and shoot apices were then removed and discarded. The remainder of the embryos were washed three times in distilled water and placed on B_5G solid medium (Gamborg et al. 1968) for 2 days at room temperature in order to initiate secondary embryo formation before they received the following separate treatments: combined microprojectile bombardment with subsequent desiccation/DNA imbibition; singular microprojectile bombardment and singular desiccation/DNA imbibition. The experiment was repeated twice for each plasmid.

Transformation protocol

For microprojectile bombardment, about 300 dissected microspore-derived targets were plated around the center of each of the 100 × 15 mm Petri dishes containing 15 ml of B_5G medium (Gamborg et al. 1968) supplemented with 0.8% agar, 0.1 mg/l gibberellic acid and 2% sucrose.

Microprojectiles (tungsten particles of 1.0 μm diameter) were prepared and DNA coating performed according to the procedures described by Klein et al. (1988). The particle gun was operated by using Dupont Biolistic PDS-1000/He Particle Delivery System (Dupont Company, Delaware, USA). Each bombardment treatment was conducted three times in order to cover all sides of the microspore-derived targets.

For the desiccation/DNA imbibition treatment, 300 dissected microspore-derived targets were evenly distributed on empty, sterile 100 × 15 mm Petri dishes. Petri dishes were left open and placed against the bench wall at an angle of about 45° in a flow bench (Canadian Cabinets, Johns Scientific, Calgary) with an exhaust requirement of 0.57 m^3/s and a temperature of 20 °C for 25 min. These microspore-derived targets were then rehydrated for 20 min in plasmid DNA solution at a concentration of 100 ng/μl.

After bombardment and/or imbibition, the microspore-derived targets were plated onto 100 × 15 mm Petri dishes containing 15 ml of the same B_5G medium (Gamborg et al. 1968) as used above, at a density of ten embryos per Petri dish. They were allowed to develop in a growth cabinet with a temperature regime of 25/19 °C (day/night) and a 16-h photoperiod with a light density of 270 $\mu Em^{-1} s^{-2}$.

Histochemical analysis of β-GUS expression

Thirty hours after treatment, the hypocotyls were analyzed for β-GUS activity using the histochemical assay described by Jefferson (1987) but with 28% methanol (v/v) in the reaction buffer to inhibit endogenous *B. napus* β-GUS activity which may mask the activity originating from the introduced GUS gene (see Kosugi et al. 1990).

NPTII transformant plant regeneration

Microspore-derived targets bombarded and/or imbibed with plasmid pALL NPT II were re-suspended for 3 days in fresh Lichter's medium supplemented with 50 mg/l of kanamycin before transfer to solid medium. Plantlet regeneration from hypocotyls was induced on fresh, selective solid B_5G medium (Gamborg et al. 1968) supplemented with kanamycin to a final concentration of 100 mg/l. Twenty hypocotyls were plated onto each 15 × 60 mm Petri dish, containing about 15 ml of solid selective medium, for further growth in a growth cabinet with a temperature regime of 25/19 °C (day/night) and a 16-h photoperiod with a light density of 270 $\mu Em^{-1} s^{-2}$.

PCR and Southern analyses

Surviving plantlets were transplanted to soil. At about the three-leaf age, using the lid of a 1.5-ml microcentrifuge tube, a leaf disc was punched from each plantlet for DNA extraction for PCR analyses (Edwards et al. 1991) using NPTII-specific primers. These plants were allowed to grow until they reached a five- or six-leaf stage, at which point all plants which were proved NPTII-positive by PCR analyses were subjected to large-scale DNA extractions for Southern analyses (Rogers and Bendich 1988). The genomic DNA of plants was digested with the restriction enzymes *Hin*dIII and *Eco*RV to generate an array of random DNA fragments. Methods used for Southern hybridi-

zation and autoradiography were those described by Sambrook et al. (1989) with a few modifications. A probe specific for the NPT II gene was prepared using the PCR product of a 700-bp fragment of the NPT II gene, with plasmid DNA as a template. The PCR product was purified and labelled with radioactive ^{32}P, using the random primed DNA labelling method provided with the labelling kit (Boehringer Mannheim).

Progeny test

Confirmed R_0 transgenic plants were colchicine doubled (Coventry and Kott 1988) and were subsequently self pollinated to provide R_1 progeny. A sample of R_1 progeny was subjected to kanamycin selection on germination media and Southern analyses were used to determine whether transgenes in the recovered R_0 plants were homozygous or heterozygous.

Results

Histochemical expression of the β-GUS gene was observed in the hypocotyls of microspore-derived embryos in *B. napus* following both the combined treatment and the singular microprojectile bombardment, but not in the singular desiccation/DNA imbibition or in the untransformed control. The percentage of hypocotyls showing β-GUS activity and the number of expression sites per hypocotyl vary with the treatments. The combined treatment yielded a higher percentage of hypocotyls expressing the introduced gene than the singular microprojectile bombardment, a four-fold increase in efficiency in comparison to the latter treatment (Table 1). β-GUS expression was observed throughout the entire length of the hypocotyls, which contains potential secondary embryogenic sites (Fig. 1). The combined treatment also produced more hypocotyls showing multi-location β-GUS expression than either separate treatment (Table 1). One-third of the hypocotyls in which β-GUS activity was observed showed multi-site (> 2 sites) expression, while only one-fifth of the responding hypocotyls was observed in the singular microprojectile bombardment treatment (Table 1). Such multi-site expression increases the likelihood of transgenic shoot apex regeneration and foreign gene integration.

In order to investigate the effect of desiccation/DNA imbibition treatment in the combined treatment, hypocotyls were treated with microprojectiles that were not coated with DNA and then subjected to desiccation/DNA imbibition. The results showed that, although this treatment also yielded transient expression, it had a lower efficiency of only one-tenth that obtained following singular microprojectile bombardment with DNA coating (data not shown). In contrast, no β-GUS gene expression was observed following the singular desiccation/DNA imbibition treatment (Table 1).

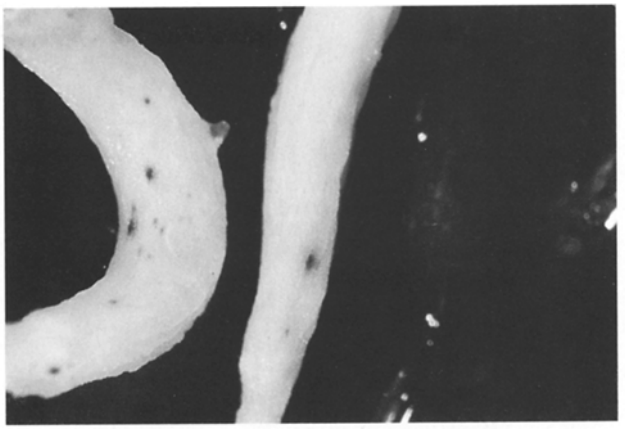

Fig. 1 Expression of the β-GUS gene in isolated hypocotyls (indicated by *dark spots* which are blue in a colored background) of microspore-derived embryos of canola (*B. napus*) following the combined treatment of microprojectile bombardment and desiccation/DNA imbibition

The results of screening plants regenerated from hypocotyls transformed with the NPT II gene on a kanamycin-containing medium is shown in Table 1. The same trend as for β-GUS gene expression was observed for NPT II gene expression. Approximately three-times as many plants survived on kanamycin medium following the combined treatment compared with the singular microprojectile bombardment treatment, although the absolute survival rate was very low (Table 1). PCR was conducted on plants regenerated from hypocotyls growing on kanamycin-containing medium. All except one of these putative transformants were PCR positive (Fig. 2). These plants were subsequently allowed to develop in soil for later Southern analysis. As expected, DNA hybridization results indicated that integrative transformation had taken place in PCR-positive plants (Fig. 3). Furthermore, the number of inserts integrating appears to be independent of the methods used.

Progeny analysis of NPTII transgenic plants was conducted on selfed plants from nine transformed R_0

Table 1 Percent hypocotyls showing β-GUS activity and percent plantlets regenerated from hypocotyls on kanamycin-containing media following microprojectile bombardment, desiccation/imbibition and the joint methods

Methods	%Embryos with β-GUS activity	%Embryos with multi-site GUS expression	%Plantlets surviving kanamycin selection
Combined method	37.4 ± 9.4	12.4	2.0
Bombardment	8.7 ± 2.1	1.7	0.6
Desiccation and imbibition (D)	0.0	0.0	0.0
Untreated control	0.0	0.0	0.0

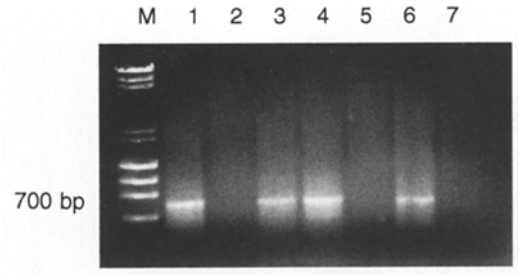

Fig. 2 PCR results from some of the putative NPTII transformants. *M*, DNA fragment size marker; *Lanes 1–4*, putative transformants; *5*, nontransformed plant control; *6*, plasmid control; *7* reagent control (without DNA template)

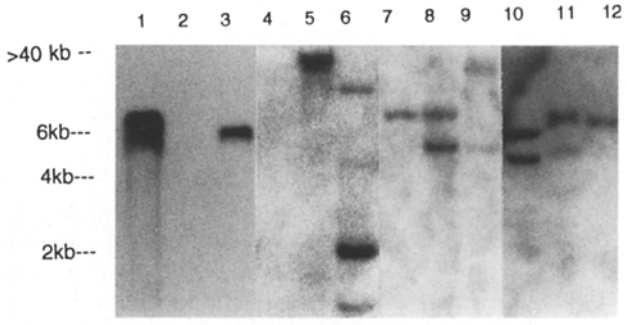

Fig. 3 Southern hybridization of putative NPTII transformants. *Lane 1*, 100 ng of plasmid DNA; *2*, 10 pg of plasmid DNA; *3*, 100 pg of plasmid with 10 µg of untransformed plant DNA; *4*, 10 µg of genomic DNA from untransformed plant control; *5–6*, 10 µg of undigested and digested (with *Hin*dIII) genomic DNA from a putative transformant; *7–9*, 10 µg of genomic DNA each of three individual transformants digested with *Hin*dIII; *10–12*, 10 µg of genomic DNA each of the same three individual transformants as in *lanes 7–9* but digested with *Eco*RV

Table 2 Progeny segregation of transgenic plants carrying the NPTII gene in canola (*B. napus*) determined by kanamycin medium screening and Southern analysis

R_0 plant #	Segregation ratio	
	kan + /kan −	(+/− by Southern)
k1	1:0	1:0
k2	1:0	1:0
k3	1:0	1:0
k4[a]	12:5	12:5
k5	1:0	1:0
k6	1:0	1:0
k7[b]	11:4	11:4
k8	1:0	1:0
k9	0:1	1:0

[a] k4 is a spontaneous diploid plant. The Chi-square test indicated that the segregation followed a 3:1 ratio
[b] k7 is a colchicine-doubled haploid. The segregation could be due to chimeric transformation

Table 3 Comparison of regeneration frequency of primary microspore-derived embryos and the isolated hypocotyls in canola (*B. napus*)

Embryo age	Percent regeneration	
	Microspore embryos	Hypocotyls
30	12 ± 2.6	80 ± 4.2
40	26 ± 3.5	88 ± 2.5
50	30 ± 2.7	96 ± 2.2
60	28 ± 1.8	82 ± 3.4

Discussion

plants, using both phenotypic scoring and Southern detection (Table 2). Two primary transformants displayed a segregation pattern in their progeny, whereas no segregation was observed in progeny lines produced from the other seven transformed plants. One progeny line was observed to be susceptible to kanamycin and Southern analysis confirmed the presence of the gene component in the plant.

Secondary embryo initiation by removal of cotyledons and shoot apices from primary embryos greatly enhances the frequency of plant regeneration. In most of the isolated embryos, hypocotyls develop multiple embryo/shoot apices that originate from the epidermal or the cortical cell layers. In this study, the average regeneration frequency of the isolated hypocotyls was close to 90% whereas that of the primary microspore-derived embryos was about 25% (Table 3). Almost every hypocotyl was observed to contain multiple shoot primordia. This high potential for plant regeneration associated with hypocotyl tissues has provided a reliable culture system for plant transformation using the haploid approach.

Transient expression of the β-GUS gene and integrative transformation of the NPT II gene in canola was observed in the current study. The combined treatment of microprojectile bombardment and desiccation/DNA imbibition greatly improved direct DNA uptake by hypocotyls of microspore-derived embryos in *B. napus* as indicated by β-GUS gene expression. Microprojectiles coated with plasmid DNA are believed to carry DNA through the cell wall and membranes, thus entering the cell, inside which the coated DNA was released from the particles (Klein et al. 1988). During the process, the microprojectiles create many tiny holes in the cell barriers.

Subsequent imbibition of DNA by desiccated cells may allow greater DNA uptake than following the biolistic treatment alone. This approach may be very similar to a microlaser method (Weber et al. 1988), in which a microlaser beam instead of a microprojectile was used to create mini-holes in the cell wall to facilitate the entry of DNA molecules. Membrane wounding seems to be a crucial factor influencing the successful introduction of foreign genes into plant cells. Without

such wounding, DNA penetration would be extremely difficult, as illustrated by our lack of success with the singular desiccation/DNA imbibition protocol and the limited success of this protocol in previous work (Chen 1991). Bidney et al. (1992) also showed that a particle gun used in combination with *Agrobacterium tumefaciens* greatly increased the efficiency of transformation of plant tissues in comparison to singular transformation protocols, presumably because microprojectile bombardment generated many wound sites for *Agrobacterium* infection.

In order to ascertain the effect of desiccation/DNA imbibition, experiments consisting of biolistic treatments using microprojectiles without DNA coating followed by desiccation/DNA imbibition were carried out. They resulted in a lower frequency of β-GUS expression sites than that obtained with singular microprojectile bombardment using DNA-coated particles (data not shown). Direct desiccation/DNA imbibition of hypocotyls yielded no transient expression of the β-GUS gene (Table 1). These observations indicate that the enhanced DNA uptake occurring with the combined method was partly due to direct desiccation/DNA imbibition only if the hypocotyls were first damaged to some extent. Nevertheless, the use of microprojectiles with DNA coating in conjunction with desiccation/imbibition provided more extensive β-GUS expression.

The use of haploid tissue targets for gene transfer provided a high frequency of homozygous transgenic plants. In the current study, seven out of nine primary transformants produced homozygous lines (Table 2). Two of the R_0 plants (k4 and k7) produced progeny lines with a 3:1 segregating ratio for kanamycin resistance. One of these two plants (k4) turned out to be a spontaneous diploid, which was considered heterozygous for the transgene. Segregation observed among k7 progeny could result from chimeric tissue transformation which distorted homozygosity.

The use of hypocotyls as targets for transformation is assumed to be advantageous over the use of primary embryos, in that hypocotyls, once detached from cotyledons, will enter a state of multiple secondary embryogenesis (Dr. L. Kott, personal communication). The occurrence of many secondary embryogenic apices increases the frequency of plantlet regeneration. Presumably, if all the secondary embryogenic apices were exposed to DNA-coated particles, the probability of regeneration of at least one transgenic plant is much greater than that of a single primary embryo. There is concern that the meristems of cereal tissues (in our case, the secondary embryogenic tissues) do not contain competent cells to receive foreign genes and to regenerate (Potrykus 1990). We did not observe such a tendency in this experiment. In fact, there have been several reports which contradicted the assumption that meristematism is incompatible with transformation competence. Successful transformation using the shoot apex has been reported by several authors (Ulian et al. 1988; Hussey et al. 1989; Schrammeijer et al. 1990; Gould et al. 1991).

The current study demonstrated a joint application of microprojectile bombardment and desiccation/DNA imbibition to microspore-derived embryo tissue targets, which greatly enhanced DNA uptake and the recovery of homozygous R_0 plants. Although the quantitative relationship between DNA uptake and the integrative events has not been elucidated, owing to the unknown mechanism of gene integration in the host genome, we believe that greater DNA uptake enhances the possibility for integrative transformation.

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