Plant Cell, Tissue and Organ Culture **40:** 97–100, 1995.

Research note

Assessment of microinjection for introducing DNA into uninuclear microspores of rapeseed

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Received 11 November 1993; accepted in revised form 9 July 1994

Key words: Brassica napus, β-glucuronidase, polymerase chain reaction, transformation

Abstract

Approximately 2,000 embryogenic uninuclear microspores of rapeseed (*Brassica napus*) cv. Topas were intranuclearly injected with a chimaeric β-glucuronidase (*Escherichia coli Uid* A) gene. Stable integration had not occurred among 55 plants that were regenerated. Coinjection of the dye Lucifer Yellow and detection of injected DNA by the polymerase chain reaction revealed high frequencies of transfer. However, the amount of DNA injected was less than 20 copies, which may have been insufficient for stable transformation of microspores.

Abbreviations: PCR – polymerase chain reaction, GUS – β-glucuronidase

Techniques have been developed for the microinjection of macromolecules into regenerable plant cell types such as protoplasts (Crossway et al. 1986; Reich et al. 1986a), suspension culture cells of carrot (*Daucus carota* L.) (Nomura & Komamine 1986), cultured microspores of rapeseed (*Brassica napus* L.) (Miki et al. 1989), barley (*Hordeum vulgare* L.) (Bolik & Koop 1991) and corn (*Zea mays* L.) (Gaillard et al. 1992) as well as microspore-derived proembryos (Neuhaus et al. 1987). Microinjection may provide an alternative transformation procedure for species in which transgenic plants are difficult to generate by more common processes that employ *Agrobacterium* or particle bombardment (Bolik & Koop 1991). Very high transformation frequencies (> 6%) have been achieved by microinjection-mediated transformation of plant cells; however, the technology has only been successfully applied to regenerating protoplasts (Crossway et al. 1986; Reich et al. 1986a) and microspore-derived embryos at the 4–12 cell stage (Neuhaus et al. 1987). In this study, DNA was introduced into uninuclear microspores of *B. napus* cv Topas by microinjection. Although the majority of visually injected microspores contained the injected DNA fragment, the amount of DNA transferred was much lower than expected and may have been insufficient to achieve stable transformation.

Uninuclear microspores were isolated from rapeseed cv. Topas buds, 3.5 to 4.3 mm in length using procedures detailed by Huang & Keller (1989). For microinjection, the microspores were immobilized in 0.4% agarose in 1/2 NLN-13 medium plated onto a ring (1.0 to 1.5 mm in depth) containing a previously solidified layer of 0.8% agarose in 1/2 NLN-13 medium (Miki et al. 1989). Ring units were placed on feeder cultures of freshly isolated microspores in 60 × 15 mm Petri dishes with 12,000 to 18,000 microspores per culture dish. They were incubated in the dark at 32.5 °C for up to three days before microinjection.

The microinjection procedure was as previously described (Miki et al. 1989) with some modifications. After washing in acetone, microinjection needles (BF100–58–10, Sutter Instruments, San Rafael, CA) were pulled on a David Kopf Instruments model

DKI 700 C pipette puller, and loaded by backfilling along their inner filament. The holding pressure on the automatic microinjector (Eppendorf Model 5242) was increased to 200 hPa from 100 hPa because we were concerned that the turgor pressure inside the microspore might be large enough to cause backfilling of the microinjection needle as it was inserted into the microspore. A pressure gauge inserted into the line between the microinjector and the needle revealed only minor changes in the holding pressure, likely due to slight differences in the openings of the microinjection needles. No pressure changes occurred when the needles were inserted into the microspores. Injections were performed with a pressure of 500 hPa for 2 seconds.

The vector pBI221.2, which contains the CaMV 35S promoter, the β-glucuronidase (GUS; *Uid* A) coding region, and nopaline synthase (*nos*) terminator (Jefferson et al. 1987) was used in all experiments. The DNA solutions contained Lucifer Yellow and were diluted in TE buffer (10 mM Tris pH 7.0, 0.1 mM EDTA) to a final DNA concentration of 30 $\mu g\ ml^{-1}$ and 2% Lucifer Yellow. The solution was centrifuged at 12,000 rpm, 30 min in a microfuge and the top 10 µl removed and transferred to a fresh microfuge tube for loading the microinjection needles. Higher concentrations of DNA resulted in more frequent plugging of the needles.

Individual microspores were removed from the 0.4% agarose using a micropipette with a large tip and placed in a 15–20 µl drop of 1/2 NLN-13 medium. DNAse 1 was added to the drop to give an enzyme concentration of 50 $\mu g\ ml^{-1}$ and the microspore was placed at 37 °C for 45 to 60 min. The microspore was then transferred to 20 µl of lysis buffer (10mM Tris pH 8, 0.1 mM EDTA, 50 mM NaCl) and boiled for 5 min. The DNA in the sample was amplified by the polymerase chain reaction (PCR) using primers located at position 400–421 and 1007–1029 of the GUS coding region.

Competitive PCR (Gilliland et al. 1989) was performed using various ratios of the pBI221.2 plasmid DNA to the competitor plasmid pBI221.1.BH1. pBI1221.1.BH1 was created by replacing the GUS gene in pBI221.1 by the full-length GUS gene PCR-amplified from the *Escherichia coli* DH5α strain. The unmodified GUS gene in DH5α contains a *Bam*HI site, which was removed during the construction of the pBI vectors. The $dCTP^{32}$-labelled PCR products were digested with *Bam*HI and electrophoresed on 0.9% agarose gels. To determine the proportion of the PCR products derived from pBI221.2 and from pBI221.1.BH1 the bands were excised and the amount of isotope incorporated into each band was measured.

In this study, about 2,000 microspores were injected intranuclearly (Fig. 1a) with the plasmid pBI221.2. From a sample of seven embryos that developed in culture, 20–30 days after injection, two showed fluorogenic GUS activities that were at least 10 fold greater than those for uninjected embryos (data not shown). Fifty five plants were regenerated and analyzed for GUS gene integration by Southern blot analysis. None were stably transformed. Experiments were therefore undertaken to examine the transfer of the DNA solution into the microspores and to determine the approximate amount of DNA injected; however, efforts were not made to ensure that subsequent injections were into the nucleus.

The presence of DNA solutions containing Lucifer Yellow was confirmed by fluorescence microscopy (Fig. 1b, 1c). Among the injected microspores the degree of fluorescence varies greatly but it was generally very weak (Fig. 1b, 1c). The microspores remained fluorescent for many days. The PCR primers directed to the GUS gene generated a 607 bp fragment (Fig. 2a, 2b) after 30–60 cycles, in the presence of 2% Lucifer Yellow. Fluorescence microscopy revealed that DNA solution was also released into the culture medium between injections during operations such as the clearing of pipette tips or changing of pipettes. In the medium, the DNA appeared to adhere to uninjected microspores and could be detected after two days by PCR (data not shown). This problem was largely resolved by collecting individual microspores in a drop of medium, treating with 50 $\mu g\ ml^{-1}$ DNAse for 1 h at 37 °C prior to lysis and analysis (eg. Fig. 2a, lanes 1–9). A total of 66 separate analyses were performed, 40 on visually-injected and DNAase-treated microspores, and the remainder on uninjected microspores which were exposed to DNA (by injecting DNA into the medium close to the microspore exine) during the experiment and then DNAase treated. The GUS gene was detected in 63% of injected microspores (eg. Fig. 2a, lanes 1 to 5). In 27% of uninjected control microspores (Fig. 2a, lanes 6–9) GUS DNA was not completely removed from the exterior and could be detected (eg. Fig. 2a, lane 8). GUS DNA was not found in experiments where the microspores were not exposed to the DNA solution. The data was consistent with the transfer of plasmid DNA in a high proportion of injected microspores.

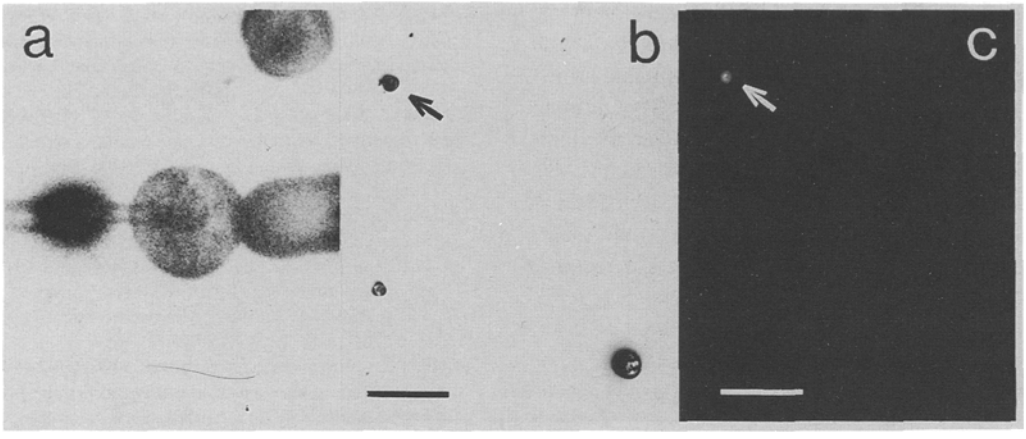

Fig. 1. Intranuclear microinjection of an embryogenic uninuclear microspore (*a*) and microspores two days after microinjection of Lucifer Yellow (*b, c*). In panel a, the pipette on the right was used to prevent microspore movement as the injection pipette on the left was inserted. After culture for 2 days in ring units placed at 32.5 °C in the dark, microspores were injected with a mixture of pBI221.2 plasmid DNA (30 ng μl^{-1} in TE) and 10% lucifer yellow. Panel b shows 3 injected microspores under bright field optics. Panel c shows the same microspores using fluorescent optics. The microspore with the greatest fluorescence is indicated by the arrow. In panels b and c the bar = 100 μM. In panel a the microspore has diam = 18 μM.

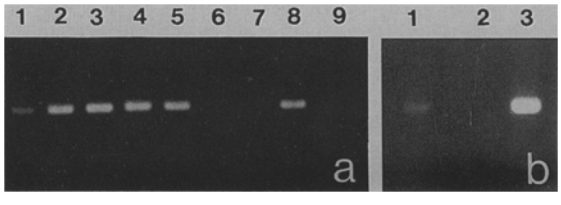

Fig. 2. (*a*) GUS DNA fragments amplified from single injected microspores (lanes 1–5) and uninjected microspores exposed to DNA externally (4 per lane; lanes 6–9). Each sample was treated with 50 μg ml^{-1} DNAase, 1 h, 37 °C. (*b*) GUS DNA fragments amplified from 10 microspores derived from transgenic rapeseed cv. Westar (lane 1) and pBI221.2 plasmid DNA (lane 3). Lane 2 is a negative control for the PCR reaction buffers and contains no DNA sample. The fragments in both (a) and (b) were generated through 50 cycles of amplification and separated on a 0.9% agarose gel stained with ethidium bromide.

The conditions of microspore collection and analysis were sufficient to detect the GUS gene in 10 microspores from transgenic rapeseed cv. Westar stably transformed by *Agrobacterium*-mediated transformation (< 25 copies of the GUS gene in total, Fig. 2b, lane 1). The possibility was therefore considered that the amount of DNA injected was much lower than previously anticipated. To estimate the copy number of injected GUS genes, the competitive PCR procedure was adopted (Gilliland et al. 1990) using the coding region of the wild type GUS gene as competitor. The results indicated that less than 20 copies of the GUS gene were injected into each microspore.

Embryogenic uninuclear microspores are relatively small in rapeseed (approximate diameter 20 μm Fig. 1), and they are contained within a rigid cell wall structure under high osmotic pressure. Although the Eppendorf microinjector was adjusted for the maximum injection volume that did not cause visual damage, it is likely that the injected volumes were much smaller than with protoplasts. We observed that the intensity of Lucifer Yellow fluorescence in microspores was much lower than for protoplasts. The elasticity and porosity of the regenerating protoplast cell membrane facilitates the injection of relatively large volumes of aqueous solution (Reich et al. 1986b) therefore thousands of plasmids could potentially be injected. High frequencies of transformation by microinjection have been reported for microspore-derived proembryos (Neuhaus et al. 1987). It is possible that the changes accompanying microspore embryogenesis and embryo formation permit the injection of larger volumes. Other studies with corn microspores (Gaillard et al. 1992) indicate that the multinucleate stage is more suitable than the uninucleate stage for microinjection of dye solution.

The amount of free DNA that must be injected to achieve transformation has not been measured directly for any system; however, previous studies have shown that a large percentage of cells (6–51%) that receive DNA in the nucleus or cytoplasm by microinjection become stably transformed (Crossway et al. 1986; Reich et al. 1986a; Neuhaus et al. 1987). In this study, DNA transfer was achieved with relative-

ly high efficiency but seemed to be insufficient to obtain stable transformation. The small amount of free DNA transferred may have been a significant limiting factor. Therefore, the development of conditions that increase the amount of DNA transferred may yield more promising results. It may be important to consider the packaging of the DNA in proteinaceous or cationic complexes to facilitate integration. At this time, there is no evidence that integration by illegitimate recombination is inhibited in these cells.

Acknowledgements

The authors wish to thank M.K. Pomeroy and N. Long for assistance in microspore isolation and J. Simmonds, D. Simmonds and W. Keller for advice and valuable comments. The research was supported by the National Research Council, Canada under the Program for Industry/Laboratory Projects Arrangement No. CA103–8–112. PRC No. 1558

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