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Transgenic rapeseed plants obtained by the microinjection of DNA into microspore-derived embryoids

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Summary. A novel method in the field of genetic engineering of higher plants is presented: microinjection into multicellular structures which have a high competence for plant regeneration through embryogenesis. Microspore-derived embryoids of *Brassica napus* L. were individually selected and microinjected with NPT II gene constructions. High frequency regeneration of haploid plants through embryogenesis was achieved within 8 weeks. Transformation efficiencies between 27% and 51% were determined by DNA dot blot analysis of primary regenerants. Stable integration of full-length microinjected genes into high molecular weight DNA was proven by Southern analysis of genomic DNA isolated from regenerated plants. Transformed plants were tested for expression of the NPT II gene by enzyme assay. The chimeric nature of the primary regenerants was demonstrated after their in vitro segregation through secondary embryogenesis into pure transformants.

Key words: Embryoid microinjection – Neomycin phosphotransferase II gene – Plant transformation – Transgenic rapeseed

Introduction

Progress in the transformation of agriculturally important crop plants has been restricted by the limitations of presently available gene-transfer systems (Potrykus et al. 1985a). Plant transformation is being routinely achieved by *Agrobacterium tumefaciens* Ti plasmid mediated DNA-transfer (Herrera-Estrella et al. 1983; Schell et al. 1984; De Block et al. 1984; Horsch et al. 1984). Unfortunately, *A. tumefaciens* DNA transfer has been limited to dicotyledons and a few non-cereal monocotyledonous species (Hernalsteens et al. 1984; Hooykaas-VanSlogteren et al. 1984).

Efficient transformation of protoplasts from a variety of plant species, including some gramineae, has been made possible by direct gene transfer of exogenous DNA (Paszkowski et al. 1984; Hain et al. 1985; Potrykus et al. 1985b, c; Lörz et al. 1985; From et al. 1985). The main disadvantage of these gene transfer methods is that they require isolated protoplasts. A reliable and efficient plant regeneration system from most cereal protoplasts is still lacking, except for rice protoplasts (Fujimura et al. 1985; Yamada et al. 1986; Toriyama et al. 1986; Hayashi et al. 1986; Coulibaly and Demarly 1986; Kyozuka et al. 1987). Therefore, novel techniques are needed to extend gene transfer to other plant species, particularly cereal crop plants. In this respect, two different experimental approaches have been recently reported: DNA injection into young floral tillers of rye (de la Pena et al. 1987) and *Agrobacterium*-mediated geminivirus-infection of maize (Grimsley et al. 1987). As an alternative strategy for the high-efficient transformation of dicots and monocots we have used microinjection of vector-free DNA (Spangenberg et al. 1986a; Neuhaus et al., in preparation) into early-staged embryoids derived from mass-cultured microspores of *Brassica napus* (Lichter 1982). Haploid plants can be rapidly regenerated from the embryoids through embryogenesis. The procedure described in this paper is the first example of exogenous DNA transfer across plant cell walls into the genome of embryogenic

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cells. This provides a powerful tool for genetically engineering plants which are not accessible to *Agrobacterium* transformation because of its limited host range, and plants which cannot be regenerated from protoplasts, e.g. the cereals.

Material and methods

Microspore isolation and microinjection of individually selected embryoids

Microspores were isolated from winter rapeseed forms 4-27-4, 5-33, 5-70 and 5-71 (Kartoffelzucht Böhm, Langquaid, FRG), grown under greenhouse conditions according to Lichter (1982), and mass-cultured in modified Nitsch and Nitsch (1967) medium (Lichter 1982; Gland et al., in preparation). Embryoids were individually selected under optical control with a hand-drawn microcapillary connected to silicone tubing and transferred for microinjection to 2 µl droplets of the same medium positioned onto a coverslip (Sprangenberg et al. 1986a). Microinjection was performed as previously described (Neuhaus et al. 1984, 1986; Spangenberg et al. 1986b) by fixing the embryoids with a holding capillary and microinjecting into each cell. Exogenous DNA, pSV2neo (Southern and Berg 1982) and K5 [gift from B. Gronenborn, Max-Planck-Institut für Züchtungsforschung, Cologne; NPT II sequence under control of the CaMV 35S RNA promoter and transcription terminator of a modified pDH 51 (Pietrzak et al. 1986)] was injected as a 1:1 mixture of linearized (by cutting the plasmids outside of the inserted genes) and supercoiled molecules at 0.5 µg/µl in 50 mM NaCl, 50 mM Tris-HCl pH 7.8.

Plant regeneration from microinjected embryoids

Immediately after microinjection with pSV2neo or K5, embryoids were individually transferred into wells of a polycarbonate microculture chamber (Spangenberg 1986a) containing 500 nl modified Nitsch and Nitsch (1967) medium. After six to ten days in microculture, early-cotyledonary embryoids were transferred into 4 µl of the same medium in wells of Terasaki dishes until formation of embryogenic structures (Fig. 1d, e). After 4 weeks of individual culture without any selection pressure, these structures were transferred onto modified B5 medium (Gamborg et al. 1968) half strength macro- and micronutrients, hormone- and vitamin-free, with the addition of 50 µg/l inositol, 2% sucrose, pH 6.0, 0.9% agar and 1% activated charcoal for differentiation of shoots from secondary embryoids (Thomas et al. 1976; Fig. 1f). Rooting of regenerated shoots was induced on the same medium (Gland et al. in prep.).

Isolation of plant DNA and Southern blot analysis

Genomic DNA of regenerated plants was isolated from freeze-dried plant material according to Lichtenstein and Draper (1985). The preparation yielded high molecular weight genomic DNA (predominantly > 50 kb), which was subjected to restriction enzyme digestion and Southern blot analysis. Genomic DNA was digested with the appropriate enzymes according to the manufacturer's protocols and electrophoresed in 0.8%–1.0% agarose gels. After transfer to Hybond-N-filters (Amersham) these were hybridized with 32P-dCTP labelled DNA probes (multiprime kit, Amersham; $5-10\times10^7$ cpm) and washed as described previously (Neuhaus et al. 1986). Genomic DNA (10 µg) used for dot blot analysis was digested overnight with EcoRI and applied to Hybond-N-filters (Amersham). Dot blot hybridization analysis was performed with apparatus from Schleicher and Schüll GmbH, Dassel (FRG), according to the manufactor's manual.

Neomycin phosphotransferase II assay

The enzymatic in situ assay for neomycin phosphotransferase II (NPT II) was carried out essentially according to Reiss et al. (1984) and Schreier et al. (1985).

Results

Microculture of microinjected embryoids

Microinjection of *B. napus* microspore-derived embryoids was performed with a rate of up to 50 preselected embryoids per hour.

Embryoids in the twelve-cell-stage proved to be optimal for this purpose, allowing good optical control during microinjection of multicellular structures (Fig. 1a, b), as well as showing high survival rates and an overall efficiency until plant regeneration of more than 80% (Fig. 1c, f). Whole plants could be regenerated as early as eight weeks after microinjection of individual embryoids.

Frequency of transformation

Genomic DNA from plants regenerated after pSV2neo (NPT II under control of SV40 early region regulatory sequences) and K5 (NPT II under control of CaMV 35S RNA regulatory sequences) microinjection of embryoids and from control primary regenerants was isolated and subjected to dot blot hybridization analysis to determine the presence of NPT II sequences. For pSV2neo transformation experiments, strong hybridization signals with the NPT II insert were obtained for 83 regenerants out of 169 (Fig. 2). Although equal amounts of genomic DNA were applied to the filter, differences in the hybridization signal were observed. This could be due to either differential integration of the foreign gene or to the chimeric character of the primary regenerants. Similar results in transformation efficiency (16 positive clones out of 56) and differences in hybridization signal were obtained with genomic DNA from regenerants derived from K5 microinjected embryoids (data not shown).

Genomic analysis of regenerated transformants

In order to determine the integration patterns of the microinjected genes, genomic DNA of regenerated plants was subjected to Southern blot analysis. For this purpose, genomic DNA was isolated from plants regenerated from secondary embryoids of single-cell origin derived from the primary regenerant (Thomas et al. 1976). Southern blot analysis of pSV2neo transformed plants revealed that the transferred genes are integrated into high molecular weight plant DNA

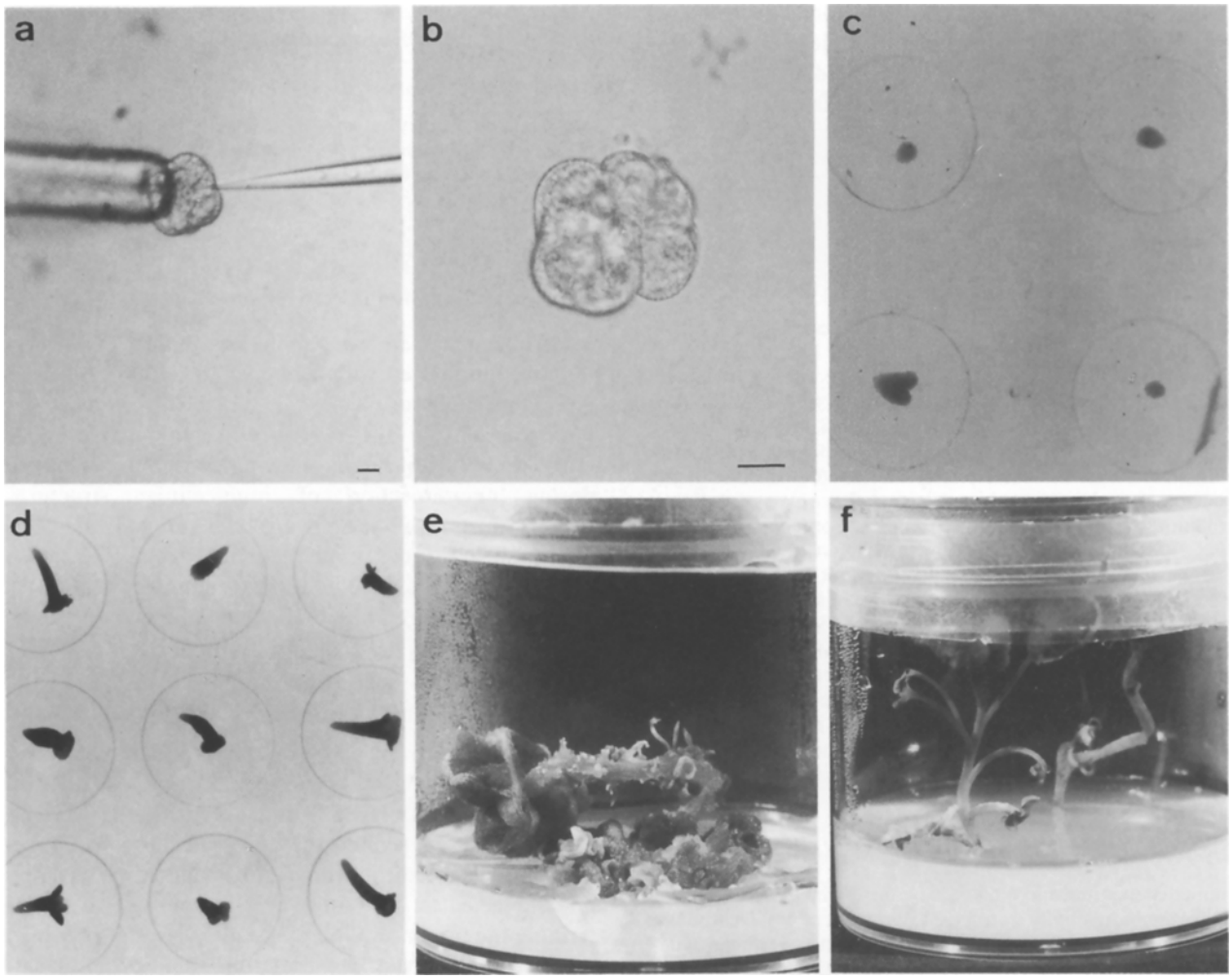

Fig. 1. **a** Microinjection into individual cells of a multicellular embryoid derived from 6 day old microspore culture of B. napus. Bar = 50 μm. **b** Detailed view of the multicellular embryoid immediately after microinjection. Bar = 50 μm. **c** Microculture of microinjected embryoids in 0.5 μl medium. **d** Individual culture of microinjected embryoids in wells of Terasaki dishes. **e** Induction of secondary embryogenesis from the primary regenerants for segregation of putative chimeras. **f** Regenerated haploid plant 8 weeks after microinjection of the microspore-derived embryoid

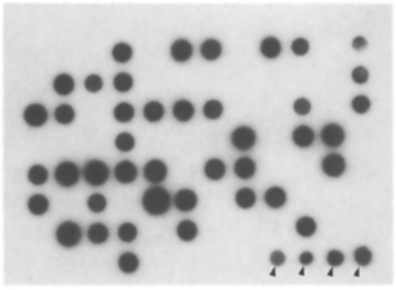

Fig. 2. Dot blot analysis of genomic DNA (10 μg each) isolated from primary regenerants after pSV2neo microinjection probed with the NPT II sequence. Positive controls (pSV2neo DNA corresponding to the amount of 1 to 3 copies per genome) are marked by *arrows*

(> 50 kb; Fig. 3a). After digestion with Taq I and Eco RI, a 2.6 kb fragment was detected, indicating that the NPT II gene together with the regulatory sequences of SV40 is integrated in the correct form (Fig. 3b). After digestion with Bcl I, for which only a single restriction site is present within the NPT II gene, Southern blot analysis revealed that tandem integration had occurred (Fig. 3c).

Similar results were obtained for the hybridization patterns of DNAs isolated from different clones derived from K5 microinjected embryoids, after digestion with Hind III, to cut out the NPT II insert of K5, probed with the NPT fragment. In most of cases Southern blot analysis showed integration of full length NPT II gene, as well as more complex patterns, indicating that several of

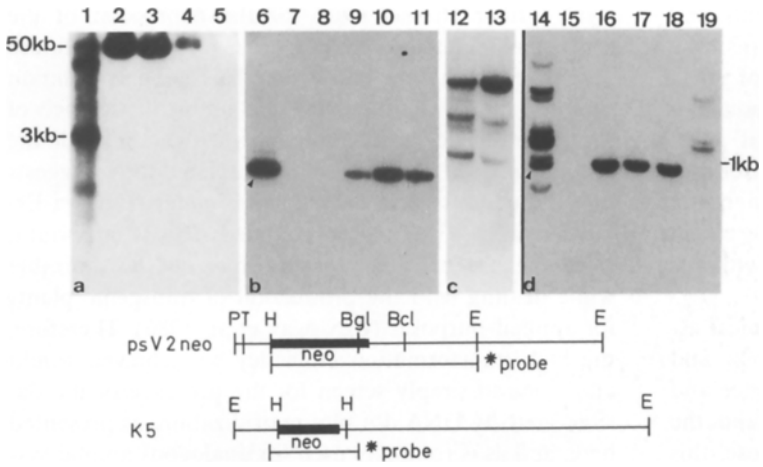

Fig. 3. Southern blot analysis (A) of undigested **a** and digested (**b** Taq I and Eco RI; **c** Bcl I; **d** Hind III) genomic DNA isolated from regenerants after microinjection of pSV2neo (**a–c**) and K5 (**d**) probed with the NPT II sequence. *Lane 1* molecular weight marker; *Lanes 2–4* and *9–13* DNA from pSV2neo microinjected transformed regenerants; *Lanes 5, 7, 8* DNA from pSV2neo microinjected, but not transformed regenerants; *Lane 6* insert from pSV2neo (0.02 ng corresponding to 4 copies per genome); *Lanes 14, 16–19* DNA from K5 microinjected transformed regenerants, *Lane 15* DNA from K5 microinjected, but not transformed regenerants. The plasmid insert and the integrated full length inserts are marked by arrows. Restriction map of pSV2neo and K5 (Bcl = Bcl I, Bgl = Bgl 1, E = Eco RI, H = Hind III, P = Pvu II, T = Taq I)

the restriction fragments contain at least part of the NPT II coding region (Fig. 3 d).

Expression of microinjected NPT II gene in transformed clones

In order to assess the expression of the transferred NPT II gene in the transformed clones, embryogenic structures and regenerated plants were assayed for the enzymatic activity of the neomycin phosphotransferase II (Reiss et al. 1984; Schreier et al. 1985). Bacterial enzyme and plant tissue derived from embryoids which had not been microinjected, as well as from those which had been microinjected but were not transformed, were used as controls. No NPT II activity was detected in either control, whereas neomycin phosphotransferase II activity could be demonstrated in all transformed embryogenic structures which were analysed, as well as in plants regenerated from them. Expression data is presented for K5 (Fig. 4); similar results were obtained for pSV2neo (data not shown).

Chimeric character of primary regenerants and in vitro segregation of putative chimeras

The fact that multicellular structures (microspore-derived embryoids in 4–12 cell or even later developmental stages) were microinjected, promoting the analysis of the putative chimeric structure of transformed regenerants. In vitro segregation of the primary regenerants was achieved by exploiting the potential for secondary embryogenesis of the well-characterized anther culture, or microspore culture-derived stem embryo system of rapeseed (Thomas and Wenzel 1975; Lichter 1982). Individual secondary embryoids considered to be of single cell origin (Thomas et al. 1976) were randomly selected from the primary regenerants and, after in vitro cloning, analyzed by dot blot hybridization for transformation. Preliminary results in this direction indicated that most of the primary regenerants

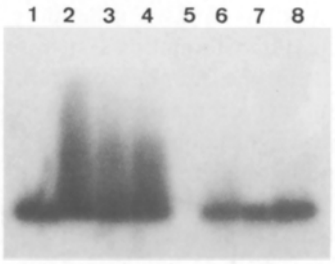

Fig. 4. Assay for NPT II activity in plants regenerated after K5 microinjection. *Lane 1* bacterial enzyme; *Lanes 2–4, 6–8* plants with integrated NPT II sequences; *Lane 5* microinjected, but no hybridization with NPT II DNA

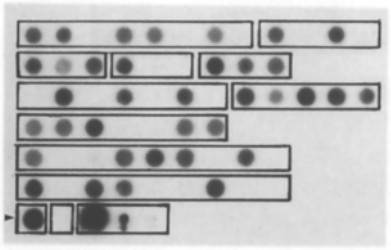

Fig. 5. Dot blot analysis of genomic DNA isolated from plants regenerated from randomly selected secondary embryoids of pSV2neo microinjected primary regenerants, probed with the NPT II sequence. Dots with DNA from individual secondary embryoids derived from one primary regenerant are *framed.* Positive and negative controls are shown in the last lane (*arrow*)

were chimeric, while in three out of ten all of the secondary embryoids tested were transformed (Fig. 5).

Discussion

A novel method for the genetic engineering of higher plants is presented: microinjection into multicellular structures which have a high competence for plant regeneration through embryogenesis. This technique rep-

resents a viable alternative to established methods, and theoretically has a broader range of applications. The method combines a transformation efficiency of up to 60%, which has been reported for microinjection into plant protoplasts and karyoplasts (Melnikov et al. 1985; Crossway et al. 1986; Reich et al. 1986; Spangenberg et al. 1986; Neuhaus et al., in prep.) with the regeneration capacity of embryogenic structures and which results in high numbers of transformed haploid plants within a short time.

Because of recent developments in the technical aspects of microinjection of plant cells (Steinbiss and Stabel 1983; Neuhaus et al. 1984, 1986; Lawrence and Davies 1985; Morikawa and Yamada 1985) and the availability of commercial setups for this purpose, this technique is no longer an extremely expensive gene-transfer system restricted to skilful hands. Furthermore, it represents the most efficient transformation system so far available with respect to the number of transformants obtained per number of manipulated cells (routinely 10%–30%). The microinjection method allows for transfer of foreign material into protoplasts (Steinbiss and Stabel 1983; Morikawa and Yamada 1985; Reich et al. 1986), cells (Nomura and Komamine 1986), or into cells of multicellular structures (this report) under controlled conditions at rates of up to 100 cells/h (Spangenberg et al. 1986 b).

Our observation, that not only early-staged embryoids of *Brassica napus* but also later developmental stages (until heart-shaped embryos) can be successfully microinjected, suggests that the same technique might also be suitable for the transformation of zygotic embryos. Methods for the isolation of zygotic embryos of rapeseed and for their cloning have been reported (Crouch 1982; Petrova and Williams 1986). For the production of pure transformants after microinjection of zygotic embryos of rapeseed, an in vitro segregation of putative chimeras through secondary embryogenesis analogous to that of microspore-derived embryoids would be desirable.

The high frequency of transformation reported here is similar to that observed with animal eggs (Brinster et al. 1981, 1985) for the production of transgenic animals after microinjection of DNA into pronuclei of fertilized oocytes (Gordon et al. 1980; Gordon and Ruddle 1981). This procedure is a unique way to overcome the lack of totipotency and organismal differentiation problems in animal systems. Similarly, after microinjection into plant protoplasts, only transformed calli have been obtained (Melnikov et al. 1985; Crossway et al. 1986; Reich et al. 1986), with the sole exception of the production of transgenic plants of *Nicotiana tabacum* and *N. debneyi* (Neuhaus et al., in prep.).

As described in the "Results" section, all positive clones detected in the DNA dot blot hybridization that were further characterized for the expression of the transferred gene showed neomycin phosphotransferase II activity in the enzyme assay. This good correlation between the data supporting the physical presence of the microinjected gene in the plant genome with the functionality of the integrated foreign DNA suggests that the need for selectable marker genes (Herrera-Estrella et al. 1983) could be obviated. This is important, since the use of such markers may not be desirable while dealing with the production of transgenic plants for applied purposes (Crossway et al. 1986). Therefore, the high transformation efficiency we achieved would allow one to simply screen for the presence of the desired gene by DNA dot blot hybridization, as presented here, and as is routinely used for analogous animal systems (Brinster et al. 1985).

Regarding the structure of the integrated DNA within the genome of the transformants, evidence is presented for both tandem integration formation [which corresponds well to previous observations of similar integration patterns when *A. tumefaciens* Ti plasmid was used as a vector (Zambryski et al. 1983)], and, in some cases, for a random integration of part or all of the injected plasmid which resulted in a more complex integration pattern. Similar complex patterns have been observed after uptake of plasmid DNA-calciumphosphate coprecipitates (Hain et al. 1985) and DNA microinjection into protoplasts (Crossway et al. 1986).

Based on our data on gene expression of the selectable marker NPT II in transgenic rapeseed plants obtained via microinjection, we are confident that microinjection into embryos will provide an excellent experimental tool for the study of gene expression and regulation during plant development, particularly during embryogenesis.

Furthermore, it should become an efficient means for producing genetically improved plants. The potential of this technique for crop improvement, especially for the recalcitrant cereals, is quite high, given the availability of different protocols for the production of embryos (that could be subjected to microinjection) through anther culture, microspore culture and somatic embryogenesis. Such protocols exist among others for maize (Lu et al. 1983), wheat (Bajaj 1984; Datta and Wenzel 1987), and rice (Siva Reddy et al. 1985). Thus, there is a good possibility that the method described in this paper might provide a universal solution to the problem of cereal transformation. Work in this respect is in progress.

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