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Haploid transformation in *Brassica napus* using an octopine-producing strain of *Agrobacterium tumefaciens*

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Summary. Microspore-derived embryos of *Brassica napus* were transformed using the disarmed octopine-producing LBA4404 strain of *Agrobacterium tumefaciens* containing the binary vector pBin19. Octopine-producing strains have previously been reported to be ineffective in transforming *Brassica*. Four actively growing yellow/green sectors were selected from the embryos on 50 mg/l kanamycin and plants regenerated. Analysis for NPT-II activity in these young plants initially indicated no expression of the bacterial *NPT-II* gene. The plants were nevertheless grown to maturity, selfed and S_1 seed was collected. Three of the S_1 plants produced microspores which were from 4 to 20 times more tolerant to kanamycin than the original parent. Southern analysis revealed that one plant (EC-1) had a single site of insertion and the other two plants (EC-2 and EC-6) had two sites of insertion with sequence homology to the bacterial *NPT-II* gene. Microspores from the EC-2 and EC-6 transgenics produced embryos on approximately five times the level of kanamycin tolerated by microspores from untransformed plants, while the EC-1 transgenic produced microspores with more than 20 times the tolerance to kanamycin. Analysis of S_1 progeny of the EC-1 transgenic indicated that 100% of the progeny exhibited the trait through both Southern analysis and by expressing tolerance to kanamycin in microspore-derived embryos.

Key words: Haploid – Transformation – Canola – Octopine – *Agrobacterium tumefaciens*

Introduction

The microspore system in *Brassica* produces large numbers of haploid embryos (Swanson et al. 1987; Swanson 1989), which have successfully been used to produce herbicide-resistant plants through mutagenesis and selection (Swanson et al. 1988). The advantages of the haploid system for the genetic modification of plants are reviewed in these reports. One of the principal advantages of haploidy for transformation is that the transgenic will have the transformed trait immediately fixed (homozygous upon colchicine doubling). This fixation results in greatly simplified genetic analysis, doubles the gene dosage and significantly reduces the required breeding time to incorporate a specific trait. By reducing the copy number, haploid transformation may also improve selection for some transgenic traits.

The nopaline synthase (nos) promoter has been used extensively for transformation in plants and was initially considered constitutive. However, it has recently been reported that the expression of the nos promoter varies widely between different tissues and organs and is also developmentally regulated (An et al. 1988). These authors concluded that the strong developmental regulation of the nos promoter may pose problems in selecting and identifying transformants in some species. *Agrobacterium*-mediated transformation of *Brassica napus* has resulted in transformed plants (Fry et al. 1987; Charest et al. 1988; Pua et al. 1987; Radke et al. 1988). However, considerable difficulty has been encountered with transforming *B. napus* using octopine-producing strains, and most studies report no success (Holbrook and Miki 1985; Pua et al. 1987; Charest et al. 1988) or very limited success (Fry et al. 1987) using these strains. Transformation frequencies in *B. napus* are generally lower and expression is weaker than observed for other species such as tobacco. The most successful transformation frequencies in *B. napus* have been reported by Fry et al. (1987) using a nopaline-producing *Agrobacterium* strain with a 35S promoter. The transformation frequencies varied from a

high of 21% with one strain and an average of 7% across strains. These authors also reported that transformation could be achieved using the nopaline synthase promoter and with at least one octopine-producing strain. However, no details on the latter transgenics were provided, except to acknowledge that the octopine-producing strains resulted in a reduced transformation frequency.

This paper describes a haploid transformation system for *B. napus*, highlights the successful use of octopine-producing strains of *Agrobacterium* to transform *B. napus* and outlines the potential use of a very sensitive post-transformation microspore assay for detecting successful integration and expression in transgenics.

Materials and methods

The *Agrobacterium tumefaciens* octopine-producing strain, LBA4404 (Hoekema et al. 1983), was used with the binary vector, pBin19 (Bevan 1984). The pBin19 vector contains a Ti region with a nopaline synthase promoter sequence in front of a neomycin phosphotransferase II (*NPT-II*) gene which confers resistance to kanamycin. A 2-day-old culture of LBA4404/pBin19 was grown in LB medium (Miller 1972) containing rifampicin (20 mg/l), streptomycin (500 mg/l) and kanamycin (100 mg/l), to maintain the binary plasmid and 20 μM acetosyringone (AS) (Aldrich, Milwaukee).

Microspore-derived embryos were produced from plants of *Brassica napus* (cv 'Topas') following the methods of Swanson et al. (1987). The synchrony of embryo development was improved by placing the petri dishes containing 14-day-old embryos on a rotary shaker (50 rpm). The donor plant conditioning and microspore isolation and culturing procedures used in this study have been recently reviewed (Swanson 1989).

Co-cultivation was initiated by transferring 100 µl of the 2-day-old LBA4404/PBin19 culture into 10 mls of the microspore medium containing approximately 600 embryos. The embryos were shaken vigorously with finely crushed glass and left for 4 h. The embryos were removed and washed twice in B5 medium, placed onto semi-solid B5 medium [0.45% agarose Type 1 (Sigma St. Louis)] containing 20 μM AS; and the co-cultivation was continued. AS had previously been identified as an inducer of virulence (vir) gene expression in octopine-producing strains of *A. tumefaciens* (Stachel et al. 1985). The petri plates were left in the light for 3 days and then washed with copious amounts of B5 medium. The embryos were dried with a paper towel and placed onto B5 medium containing 100 mg/l each of cloxacillin (Sigma), cefoxitin (mefoxin, Merek Sharp & Dohme, Toronto, Canada) and kanamycin acid sulfate (Sigma) for 2 weeks. The embryos were subsequently subcultured every 2 weeks onto B5 medium with 100 mg/l cefoxitin and cloxacillin, to control the *Agrobacterium* and alternating subcultures with 50 mg/l kanamycin.

One dark-green (EC-1) and three light-green (EC-2, EC-3, EC-6) embryo sectors grew on the kanamycin medium and were dissected out and plants regenerated by subculturing onto B5 medium (0.45% agarose Type 1) with 0.05 mg/l benzyl amino purine (BAP). Two other light-green embryos (EC-4 and EC-5) developed very slowly and have not been analyzed further. The EC-1, EC-2, EC-3 and EC-6 plantlets were transferred to Pro-Mix C (Plant Products, Toronto, Canada) and placed in the greenhouse. To double the plants and restore fertility, small plantlets with active root growth were removed, rinsed with water and placed in a beaker with 0.2% colchicine for approximately 5–6 h. The roots were then rinsed with water and the plants were repotted. Sectors with normal flower morphology were used to produce selfed seed.

Microspores were isolated from 'Topas' and from the plants derived from the selfed seed (S_1) of the four putative transformants (EC-1, EC-2, EC-3, EC-6). The microspores were plated in microspore medium containing 0, 5, 10, 20, 40, 80 and 100 mg/l kanamycin, using three isolations and two replications per level. Embryo development was recorded after 3 weeks.

NPT-II activity was assayed according to the dot blot method of McDonnell et al. (1987). A previously isolated canola transgenic with the 35S promotor was used as the positive NPT-II control.

Nuclear DNA extracted from green leaves was digested with EcoR1 and HindIII and 10 µg per sample was fractionated on 1% agarose gels in TRIS-borate buffer. The DNA was transferred by Vacugene (Pharmacia) to Gene Screen Plus (New England Nuclear) and probed with an oligolabelled (Pharmacia) *NPT-II* fragment isolated from a gel with GeneClean (Bio 101). Hybridization and washing conditions for the membrane were those recommended by the manufacturer.

The determination of whether the EC-1 insertion site was homozygous was done by testing the microspore tolerance of 12 separate S_1 plants and by performing Southern analysis of 8 other individual S_1 plants.

Results

Only one of four embryos selected (600 co-cultivated) had a strong green color on 50 mg/l kanamycin, and this embryo (EC-1) was slow to develop. The other three embryos were yellow/green in color and were selected due to their rapid growth rate on kanamycin. These four selected embryos produced double haploid plants which initially gave a negative response to the NPT-II assay from young leaf tissue. No indication of even very low NPT-II activity was evident. All the regenerated plants produced S_1 seed after colchicine doubling.

Isolated microspores of *Brassica napus* are completely inhibited by 5 mg/l kanamycin; however, selfed seed from three of the four putatively transformed double haploid plants produced plants with microspores which tolerated much higher levels of kanamycin (Table 1). EC-1 plants produced microspores which formed embryos on 80 mg/l of kanamycin, which represented more than 20 times the level tolerated by the parental material. The EC-6 microspores tolerated up to 30 mg/l, but embryo growth and development were slower than EC-1. Similarly, microspores from EC-2 tolerated 20 mg/l, but embryo development was also slow compared to EC-1. The tolerance of the microspores to kanamycin was characteristic of the particular transgenic. While embryo survival was optimum when the microspore isolations produced 200–500 embryos per dish in the untreated controls, the tolerance was evident even in isolations where only 50 embryos were produced per dish. Microspore isolations from the 'Topas' cultivar have been challenged

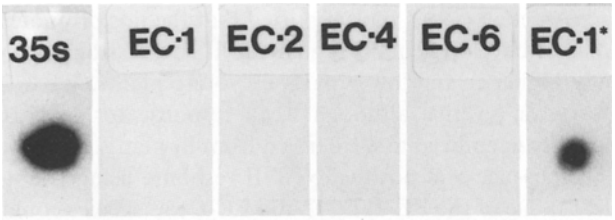

Fig. 1. NPT-II assays on leaf tissue from the S_1 progeny of four putative canola transgenics (EC-1, EC-2, EC-3, EC-6) regenerated from microscope-derived embryos following co-cultivation with LBA4404 containing the pBin19 vector. The NPT-II assay of EC-1 * was performed using microscope-derived embryo tissue of EC-1

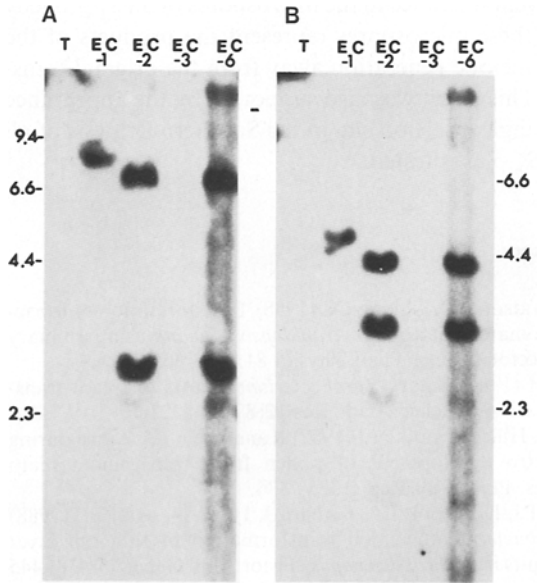

Fig. 2 A and B. Blot of nuclear DNA from 'Topas'(T) and the S_1 progeny from four putative transgenics digested with **A** EcoR1 and **B** HindIII and probed with a NPT-II fragment. The transgenics were regenerated from embryos derived from 'Topas' microspores, following co-cultivation with LBA4404 containing pBin19

Table 1. The production of microspore-derived embryos from S_1 plants of three transgenics containing the *NPT-II* gene, on increasing levels of kanamycin

	No. of embryos produced (Kanamycin mg/l)						
	0	5	10	20	40	80	100
'Topas'	550ab*	0c	0c	0c	0b	0b	0a
EC-1	410b	150a	100a	100a	20a	7a	0a
EC-2	360b	40b	20b	8b	0b	0b	0a
EC-3	400b	0c	0c	0c	0b	0b	0a
EC-6	710a	55b	35b	15b	2b	0b	0a

* Numbers within a column that are followed by the same letter are not significantly different at the 5% level according to Duncan's multiple range test

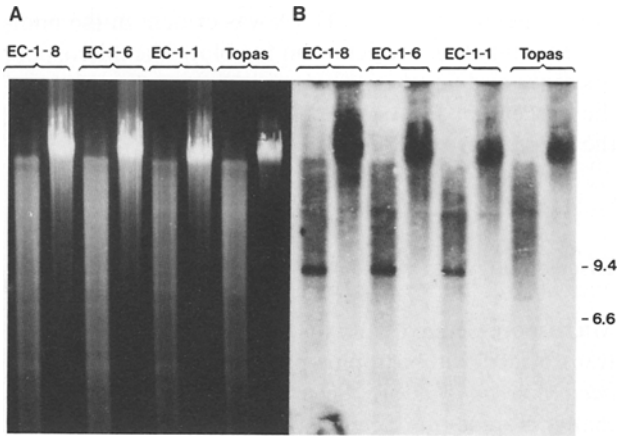

Fig. 3 A and B. Paired samples of undigested and digested (EcoR1) nuclear DNA electrophoresed on a gel **A** and probed with an NPT-II fragment in a Southern blot **B;** EC-1-1,6,8 are S_1 progeny of EC-1, a plant regenerated from a co-cultivated and kanamycin-selected microspore-derived embryo from 'Topas'

repeatedly with 5 mg/l kanamycin but have never produced embryos. The EC-3 plant produced microspores with tolerance similar to 'Topas'.

NPT-II assays and Southern analysis were performed on the S_1 plants from EC-1, EC-2, EC-3, EC-6 and 'Topas' using young leaf tissue. The NPT-II assays were negative for EC-1, EC-2, EC-3, 'Topas' and EC-6 (Fig. 1). NPT-II assays using embryo tissue derived from microspores of S_1 plants of EC-1 produced a positive NPT-II assay. The 35S transgenic used as the positive control was obtained from an earlier co-cultivation using the methods of Fry et al. (1987), and from a C58 nopaline-producing strain of *Agrobacterium tumefaciens*.

Southern analysis indicated that EC-1 plants contained *NPT-II* gene(s) which occupied a single integration site, while EC-2 and EC-6 had two integration sites (Fig. 2). There were no fragments of the size of the vector pBin19 (10,251 bp) in the HindIII or EcoR1 DNA digests (pBin19 has a single HindIII and EcoR1 site). There were also no bands of this size in lanes with undigested DNA (results not shown). The banding patterns of EC-2 and EC-6 appeared very similar, and crossing studies are under way to determine if these transformants involve the same or different loci. EC-3 which produced microspores with no increased tolerance to kanamycin was also negative for NPT-II sequences by Southern analysis.

To determine if the inserted genes were fixed (homozygous), microspores from 12 separate S_1 plants of EC-1 were extracted and were identical in kanamycin tolerance to the original EC-1 plant. Another 8 individual S_1 plants of EC-1 were analyzed by Southern analysis and each gave the same single insertion site of EC-1 (blots from three of these Southerns are shown in Fig. 3).

No unintegrated plasmid DNA was evident in the undigested samples in the gel or on the blot. The microspore tolerance and Southern analysis indicated that 100% of the progeny of EC-1 maintained both the presence and the expression of the EC-1 double haploid transgenic.

Discussion

Microspore-derived embryos of *Brassica napus* may be transformed with octopine-producing strains of *Agrobacterium tumefaciens*. Several authors have reported that octopine-producing strains do not transform *B. napus* (Holbrook and Miki 1985; Pua et al. 1987, Charest et al. 1988). We observed that transformation was occurring with the octopine-producing strain LBA4404 at a very low frequency (3 out of 600 embryos), however, many transgenics may not have been identified because of low levels of resistance to kanamycin and low enzyme activity in NPT-II assays. Microspores of *B. napus* were observed to be very sensitive to kanamycin within the first few days after isolation and cannot produce embryos if treated with 5 mg/l kanamycin. The microspores from three transgenics produced distinct survival curves on kanamycin. These curves indicated not only the stable integration of the *NPT-II* gene in the transgenics, but also provided a measure of the strength of expression in any particular transgenic. Despite a higher copy number of the *NPT-II* gene (as determined by inspection of the autoradiograph), both EC-2 and EC-6 were characteristically lower in kanamycin tolerance than EC-1. The EC-1 transgenic could be distinguished from the other transgenics by producing microspore-derived embryos more rapidly and at higher levels of kanamycin than EC-2 or EC-6. Confirmation of the transgenic nature of the microspore-tolerant selections was confirmed by Southern analysis in every case.

The kanamycin kill curves reported are consistent for microspores within the first three days after isolation, however, increasingly higher levels of kanamycin are required if multicellular embryos are used. Selection with kanamycin became increasingly more unclear and escapes are more probable as the microspores become multicellular. Microinjection of microspore-derived embryos has recently been demonstrated as an alternative method for producing haploid transgenic plants of *B. napus* (Neuhaus et al. 1987).

Contrasting results have recently been reported for the effect of kanamycin on pollen grain germination from transgenic plants. In tomato, transgenic plants have been reported to produce pollen grains with increased tolerance to kanamycin (Bino et al. 1987), while similar studies in tobacco indicated no tolerance was evident in the germinating pollen grain (Hoffmann et al. 1988).

An et al. (1988) have reported that the nos promoter may be developmentally regulated with expression generally higher in the lower parts of young plants. We observed a positive although weak response for NPT-II activity in embryo tissue derived from S_1 plants of EC-1. The absence of a positive NPT-II response using young leaf tissue of the EC-1, EC-2 and EC-6 transgenics could be interpreted as indicating that expression of the introduced genes was stronger in microspore-derived embryo tissue. Supporting this interpretation was the observation of a positive NPT-II result using embryo tissue of EC-1.

The homozygous nature and stability of the EC-1 transgenic was confirmed by the appearance of kanamycin tolerance in the microspores of all S_1 progeny tested (these microspores represent the products of the second meiotic generation away from the original transgenic). This result was also supported by the appearance of the single insertion site in the Southern analysis of all of the S_1 plants tested.

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