Theor Appl Genet (1988) 75: 685–694

Transformation of *Brassica napus* L. using *Agrobacterium tumefaciens*: developmentally regulated expression of a reintroduced napin gene

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Received September 29, 1987; Accepted October 5, 1987
Communicated by I. Potrykus

Summary. Genetically transformed plants of *Brassica napus* L. (oilseed rape) were obtained from hypocotyl explants using *Agrobacterium tumefaciens* vectors. Hypocotyl explants were inoculated with disarmed or oncogenic *A. tumefaciens* strains, EHA101 and A281, and then cultured on media containing kanamycin. The *A. tumefaciens* strains harbored a binary vector, which contained a neomycin phosphotransferase II (NPT II) gene driven by the 35S promoter of cauliflower mosaic virus and an engineered napin (seed storage protein) gene with its own promoter (300 nucleotides 5′ to the start of translation). Transformation of *B. napus* plants was confirmed by detection of NPT II enzyme activity, Southern blot analysis and inheritance of the kanamycin-resistance trait (NPT II gene) in the progeny. Expression of the engineered napin gene in embryos but not in leaves of transgenic plants was observed by Northern analysis. These data demonstrate that morphologically normal, fertile transgenic *B. napus* plants can be obtained using *Agrobacterium* as a gene vector and that developmentally regulated expression of reintroduced genes can be achieved.

Key words: *Brassica napus* – Oilseed rape – Gene transfer – Seed storage protein gene – Tissue-specific expression

Introduction

Brassica napus (oilseed rape) is the primary oilseed crop in China, Northern Europe and Canada and therefore an important target for crop improvement. The results of plant breeding efforts have greatly improved the crop (e.g., low glucosinolates, increased yield, agronomic characteristics) but genetic engineering offers new possibilities. Genetic transformation of crop plants is also an important tool for studying developmental gene regulation. There are reports of *B. napus* transformation using *A. rhizogenes* (Ooms et al. 1985; Guerche et al. 1987), but the transgenic plants had altered morphologies due to the presence of oncogenes characteristic of *A. rhizogenes*. Binary vectors and non-oncogenic *A. tumefaciens* strains have been developed to eliminate the transfer of oncogenes into plants and therefore increase the chances of regenerating transgenic plants with normal phenotypes (Hoekema et al. 1983; Fraley et al. 1986).

One important set of goals for improving the agronomic character of oilseed rape by genetic engineering involves altering the oil content and composition of seeds (Knauf 1987). Because wide variability in fatty acid content and composition is observed in *Brassica* germplasm there is reason to expect that further alterations introduced by genetic engineering would be tolerated (Mitra and Bhatia 1979). However, genetic engineering of lipid metabolism must limit its effects to the oil-storing embryo in the seed in order to avoid disrupting complex lipid biosynthesis necessary for essential membrane structures in other plant tissues such as leaves. Genes introduced to effect changes in seed lipid traits should include regulatory regions specific for gene expression during seed development. Napins are embryo-specific seed storage protein genes whose expression has been studied in *B. napus* (Crouch et al. 1983; Scofield and Crouch 1987). The regulatory signals of napin may prove useful in the expression of genes related to lipid biosynthesis.

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Recently a transformation system using *B. napus* stem explants was described (Pua et al. 1987). In this paper we describe a hypocotyl explant system for gene transfer to *B. napus* using binary, disarmed strains of *A. tumefaciens* and its use in the study of developmental regulation of an engineered napin gene in an oilseed crop.

Materials and methods

Bacterial strains, plasmids, growth conditions and transformations

Agrobacterium strain EHA101 (Hood et al. 1986) contains a C58-type chromosome (Biotype I *Agrobacterium*) with a disarmed version of pTiBo542 in which the Ti plasmid contains vir^+ genes necessary for T-DNA transfer but lacks oncogenes associated with tumor phenotypes in plant tissues (Garfinkel et al. 1981). *A. tumefaciens* strain A281 which contains the oncogenic pTiBo542 plasmid, has been described (Sciaky et al. 1978). *E. coli* strain C2110 is a polA derivitive (*pol A, his, rha*). pUC plasmids, pEMBL plasmids and their hosts have been described (Vieira and Messing 1982; Dente et al. 1983).

Agrobacterium strains were maintained on MG/L media, which is 50% Luria broth and 50% of a mannitol-glutamate-salts medium (Garfinkel and Nester 1980), containing 100 mg/l gentamicin when appropriate. For co-cultivation experiments *Agrobacterium* strains were maintained by restreaking every two weeks onto AB minimal medium (Watson et al. 1975) containing 100 mg/l gentamicin. *E. coli* strains were grown on Luria broth plates at 37 °C, supplemented when appropriate with either chloramphenicol (30 mg/l), penicillin (300 mg/l), or gentamicin (20 mg/l).

Transformation of *Agrobacterium* strains (Holsters et al. 1978) with plasmid DNA was done as follows. One-half ml of a 2 ml overnight bacterial culture was inoculated into 100 ml of MG/L broth and grown in a shaking incubator at 30° for 4–5 h. The culture was pelleted at 10,000 × g and resuspended in 1 ml MG/L broth. Cells (0.2 ml) were added to approximately 1 μg of plasmid DNA in 0.1 ml of MG/L and frozen immediately in a dry ice/ethanol bath for 5 min. The cells were thawed in a water bath at 37 °C for 5 min then grown in 2 ml MG/L broth for 2 h shaking at 30 °C. Bacteria were then plated onto MG/L containing 100 mg/l gentamicin.

Enzymes and DNA manipulations

Restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA) Reaction conditions and DNA manipulations were according to Maniatis et al. (1982). Plant DNA was isolated by the method of Dellaporta et al. (1983). Oligonucleotides were synthesized on an Applied Biosystems model 380a DNA synthesizer according to a protocol recommended by the manufacturer. Nick translation reagent kits were from BRL (Gathersburg, MD) and used according to the protocol recommended by the manufacturer to generate radiolabeled probes.

Construction of binary vector pCGN767

The construction of the binary T-DNA vector, pCGN763, and its manipulations in *E. coli* and *Agrobacterium* are described elsewhere (Houck et al., in press) and are available on request. A clone of a napin gene (pgNa) has recently been described (Scofield and Crouch 1987). The EcoRI fragment of pgNa, a 3.3 Kb fragment *B. napus* DNA containing a napin gene, was transferred to a chloramphenicol-resistant analogue of pUC18 (pCGN565). A 278 bp piece of bacterial DNA containing a dihydrofolate reductase (DHFR) protein coding region (nucleotides 804-1082 of sequence from Swift et al. 1981) was inserted into the XhoI site 10 base pairs after the stop codon of the napin gene. This bacterial sequence was expected to "tag" the napin gene so that its messenger RNA could easily be distinguished from the mRNAs of endogenous napin genes when it was reintroduced into the *B. napus* genome. DHFR sequences do not cross-hybridize with *B. napus* mRNAs under stringent conditions (see "Results"). The tagged napin gene was then transferred as an EcoRI fragment into pEMBL8. Two synthetic 27mer oligonucleotides: (5′)-CCTGATGATGATGATGCTGCAGCT-(3′) and (5′)-GCAGCATCATCATCATCATCAGGAGCT-(3′) were annealed resulting in a small piece of DNA containing a PstI site and possessing sticky ends compatible for cloning into SstI sites, but restoring an SstI site on one terminus only. This small linker was cloned into the unique SstI site in the coding region of the napin gene present in pgNa. The proper orientation was detected by PstI digests and confirmed by DNA sequencing of the tagged, engineered gene. The insert extended the coding region of the predicted napin large subunit (Crouch et al. 1983) by nine amino acid residues including 5 consecutive methionine codons. The tagged, engineered napin gene was transferred back into pCGN565 as an EcoRI fragment to create pCGN757c. pCGN757c was linearized with HindIII and cloned into a T-DNA binary vector pCGN763 (Fig. 1) by direct selection for chloramphenicol resistance in *E. coli* strain C2110 (the pCGN757c replicon is not maintained in strain C2110 unless rescued by a functional replicon such as present on pCGN763). The resulting binary T-DNA vector plasmid, pCGN767 (Fig. 1), contains between T-DNA borders: a procaryotic gentamicin marker; a eucaryotic kanamycin marker consisting of cauliflower mosaic virus (CaMV) 35S promoter, the Tn5 neomycin phosphotransferase II (NPT II) coding region, and a pTiA6 tml 3′ region (Gardner et al. 1986); and the tagged, engineered napin gene with 300 base pairs of putative promoter and approximately 2.4 kb of *B. napus* DNA found immediately 3′ to the napin gene.

Plant material and transformation

Seeds of *Brassica napus* cv. Westar were soaked in 95% ethanol for 2 min, surface sterilized in a 1.0% solution of sodium hypochlorite containing a drop of Tween 20 for 45 min, and rinsed three times in sterile, distilled water. Seeds were then plated in Magenta boxes with 1/10th concentration of Murashige minimal organics medium (Gibco) supplemented with pyridoxine (50 μg/l), nicotinic acid (50 μg/l), glycine (200 μg/l), and 0.6% Phytagar (Gibco) pH 5.8. Seeds were germinated in a Percival chamber at 22 °C in a 16 h photoperiod with cool fluorescent and red light of intensity approximately 65 μ Einsteins per square meter per second ($\mu Em^{-2}s^{-1}$).

Hypocotyls were excised from five day old seedlings, cut into pieces approximately 4 mm in length, and plated on feeder plates (Horsch et al. 1985). Feeder plates were prepared one day before use by plating 1.0 ml of a tobacco suspension culture onto a petri plate (100 × 25 mm) containing about 30 ml B5 salts and vitamins (Gamborg et al. 1968) with 3% sucrose, 2,4-D (1.0 mg/l), kinetin (1.0 mg/l), 0.6% Phytagar, and pH adjusted to 5.8 prior to autoclaving (B5 0/1/1 medium). A sterile filter paper disc (Whatman 3 mm) was placed on top of the feeder layer prior to use. Tobacco suspension cultures were subcultured weekly by transfer of 10 ml of culture into 100 ml fresh Murashige minimal organics medium

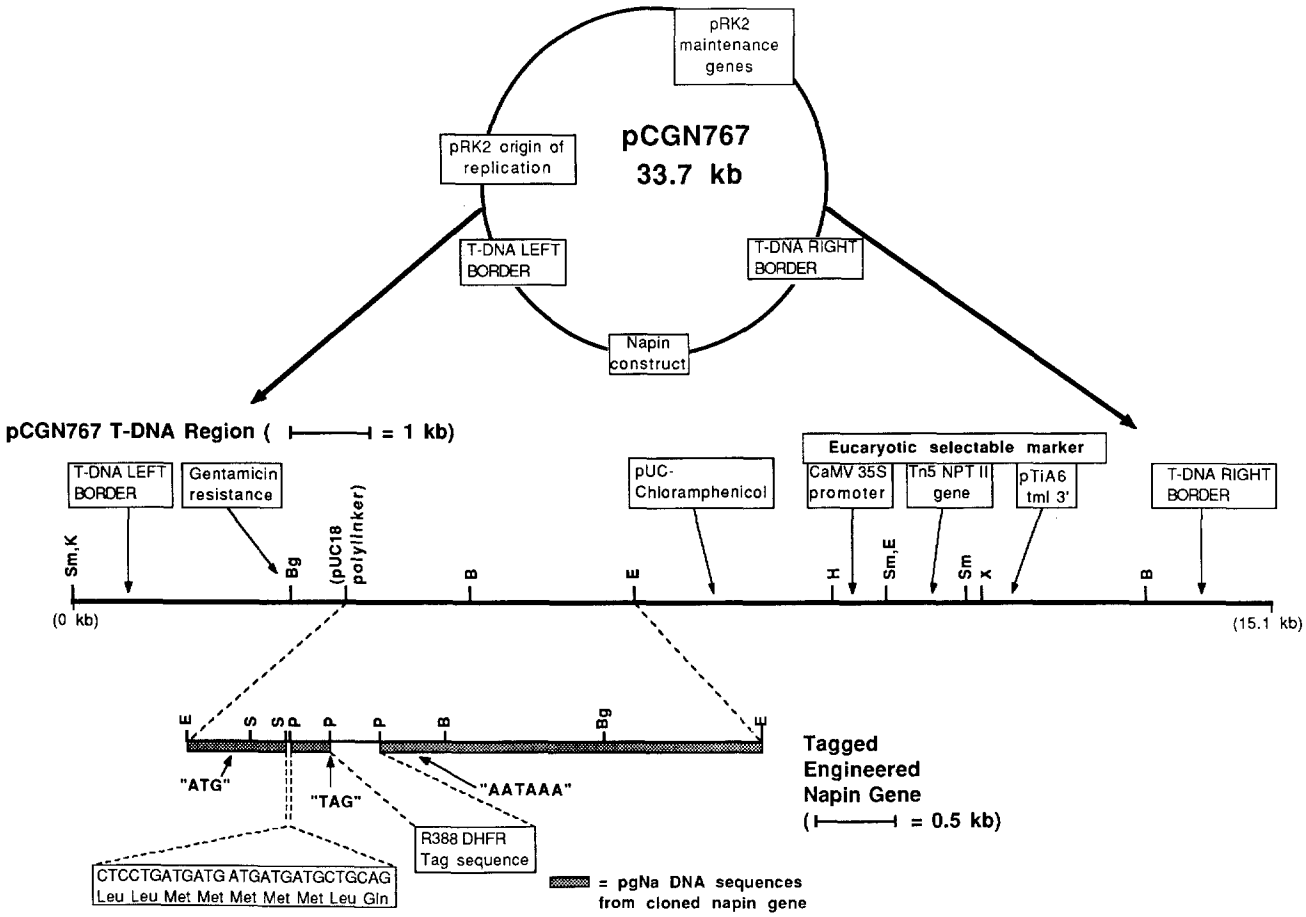

Fig. 1. Genetic and restriction map of the binary T-DNA vector pCGN767. Linear portion represents only 15.1 kb of pCGN767; the other part of pCGN767 corresponds to the 18.6 kb BglII to SalI fragment of the wide host range cloning vector pVCK102 (Knauf and Nester 1982). The junctions between the two domains are a BglII/BamHI fusion and a SalI/XhoI fusion. (Sm = SmaI, K = KpnI, Bg = BglII, B = BamHI, E = EcoRI, H = HindIII, X = XhoI, P = PstI, S = SstI)

(Flow Labs) supplemented with KH_2PO_4 (200 mg/l), thiamine hydrochloride (0.9 mg/l), 2,4-D (0.2 mg/l), and kinetin (0.1 mg/l). In experiments where feeder cells were not used hypocotyl explants were cut and placed onto a filter paper disc on top of B5 0/1/1 medium. All hypocotyl explants were preincubated on feeder plates for 24 h at 22 °C in a 16 h photoperiod of light intensity 30 $\mu Em^{-2}s^{-1}$ to 65 $\mu Em^{-2}s^{-1}$.

Single colonies of *A. tumefaciens* strains A281 and EHA 101 containing the binary pCGN767 plasmid were transferred to 5 ml MG/L broth and grown overnight at 30 °C. Hypocotyl explants were immersed in 7–12 ml MG/L broth with bacteria diluted to 1×10^8 bacteria/ml and after 30–60 min replaced onto feeder plates. After 24, 48, or 72 h of co-incubation with *Agrobacterium*, the hypocotyl explants were transferred to B5 0/1/1 callus induction medium which contained filter sterilized carbenicillin (500 mg/l, added after autoclaving). In some experiments kanamycin sulfate (Boehringer Mannheim) at concentrations of 10, 25, or 50 mg/l was added to the B5 0/1/1 medium.

After seven days in culture (conditions as described for seed germination), callus tissue was visible on the cut surface and the hypocotyl explants were transferred to shoot induction medium, B5BZ (B5 salts and vitamins supplemented with 3 mg/l benzylaminopurine, 1 mg/l zeatin, 1% sucrose, 0.6% Phytagar and pH adjusted to 5.8). This medium also contained carbenicillin (500 mg/l) and kanamycin sulfate (10, 25, or 50 mg/l). Hypocotyl explants were subcultured onto fresh shoot induction medium every two weeks.

Shoots regenerated from the hypocotyl calli after one to three months. Green shoots at least 1 cm tall were excised from the calli and placed on medium containing B5 salts and vitamins, 1% sucrose, carbenicillin (300 mg/l), and 0.6% Phytagar. Several leaves were removed from each shoot and tested for NPT II activity. Shoots which produced leaves exhibiting NPT II enzyme activity were clonally propagated and transplanted into Magenta boxes containing root induction medium (B5 salts and vitamins, 1% sucrose, 2 mg/l indolebutyric acid, 200 mg/l carbenicillin, and 0.6% Phytagar).

Plant growth conditions and sampling

The plants which expressed NPT II activity were planted in 4 inch pots containing a mixture of peat moss, vermiculite, and sand (3 : 2 : 1). They were grown in a growth chamber at 22 °C in a 16 h photoperiod with light intensity 220 $\mu Em^{-2}s^{-1}$. After about one month, plants were transplanted into 2 gallon pots

and transferred into a greenhouse for further growth. Leaf tissue from plants which expressed NPT II enzyme activity and from untransformed plants was collected and frozen at −70 °C. For expression analysis, flower buds were tagged at anthesis and seeds collected at 25 days post-anthesis. Embryos were dissected from the seeds, immediately frozen in liquid nitrogen, and stored at −70 °C.

NPT II enzyme assay

Shoots which regenerated from kanamycin-resistant calli were assayed for expression of the NPT II gene. A dot blot assay in which NPT II was detected in a crude plant homogenate was used (Moloney and Ward 1988). Leaf tissue (50–100 mg) was ground in Eppendorf tubes containing equivalent amounts w/v (50–100 µl) of extraction buffer (2.5 mM Tris pH 6.8, 0.143 mM β-mercaptoethanol, 0.27 mM leupeptin) then centrifuged for 15 min. Twenty µl of the supernatant from leaf homogenates was added to 10 µl aliquots of reaction buffers A and B. Reaction buffer A contained 67 mM Tris-Maleate, 42 mM $MgCl_2$, 400 mM NH_4Cl, 1.7 mM dithiothreitol, 0.4 mg/ml kanamycin sulfate. Reaction buffer B was identical to A except kanamycin was omitted. Five µl of ATP solution (1.0 µCi [γ-^{32}P] ATP per 5 µl, 0.75 mM ATP dissolved in reaction buffer B) was added to each reaction mixture. The samples were incubated in a 30 °C water bath for 30 min. Using a "Bio-Dot" (Bio-Rad) blotting apparatus, reaction samples were blotted onto three layers of Whatman P81 ion exchange papers placed on top of one Whatman 3 mm paper. All three P81 papers were washed twice with water (room temperature) for 4 min each. The blots were incubated for 45 min at 65 °C in 10 ml proteinase K solution (1.0 mg/ml proteinase K in 1% SDS), then washed once in 80 °C water for 4 min followed by a wash in room temperature water. The proteinase K wash was used to reduce background radioactivity caused by non-specific binding of $^{32}PO_4$ in phosphorylated proteins. After drying, blots were exposed to X-ray film with an intensifying screen. The NPT II native polyacrylamide gel assay described by Reiss et al. (1984) with the addition of the proteinase K wash described above was also used to test leaves from mature plants.

RNA analysis

Total RNA was isolated from frozen (−70 °C) leaves of transformed plants and from developing embryos dissected from seeds 25 days post-anthesis (Crouch et al. 1983). Fifty µg of total RNA was electrophoresed on a formaldehyde-containing 1.5% agarose gel as described (Shewmaker et al. 1985) and blotted to nitrocellulose (Thomas 1980). Blots were prehybridized and hybridized in 50% formamide, 0.75 M NaCl, 0.15 M Tris-HCl pH 8.0, 10 mM EDTA, 50 mM sodium phosphate pH 6.8, 1× Denhardts (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA), 0.1% SDS, 250 µg/ml salmon sperm DNA and 500 µg/ml yeast RNA, 10% dextran sulfate (hybridization only), at 55 °C. The probe was ^{32}P-labeled RNA transcribed from a SP6 vector (Promega Biotec) containing the DHFR sequence. Blots were washed for 30 min three times at 60 °C in 0.5 M NaCl, 0.025 M sodium phosphate pH 6.5, 1 mM EDTA, 0.1% SDS.

Inheritance studies

To determine how the kanamycin-resistance trait (NPT II gene) segregated in progeny, seeds were collected from untransformed *B. napus* cv. Westar and from transformed plants, surface sterilized as described above, and germinated on 1/10th MS agar medium supplemented with 100 or 150 mg/l kanamycin. After about two weeks seedlings were scored for bleaching of cotyledons and leaves and/or increased levels of anthocyanins relative to untransformed *B. napus* cv. Westar seedlings.

Results

Plant transformation and regeneration

Development of a transformation system for *B. napus* required the following: shoot regeneration from explants and optimization of co-cultivation and selection conditions. The first, shoot regeneration, was achieved from hypocotyl explants. An average of 26% of hypocotyl explants which were not co-cultivated regenerated one to three shoots each. When explants were co-cultivated with *Agrobacterium* strains EHA101 (disarmed) and A281 (oncogenic) containing the binary plasmid pCGN767 the frequency of shoot regeneration in the absence of selection was 12 and 3%, respectively. The low frequency of shoot regeneration observed with the A281 based binary is probably due to insertion of the wild type T-DNA evidenced by large numbers of kanamycin-resistant tumorous calli. Transformed and untransformed shoots reported here were obtained from explants cultured in a 16 h photoperiod for at least one month before transferring to continuous light. In subsequent experiments, hypocotyl explants cultured in continuous light regenerated about twice as many shoots as those cultured in a 16 h photoperiod.

Transformation frequency was measured as the percentage of explants which regenerated shoots on kanamycin-containing media and subsequently tested positive for NPT II enzyme activity. When hypocotyl explants were co-cultivated with *Agrobacterium* containing the binary plasmid pCGN767, the shoot transformation frequency varied from 0.4 to 2.5% (Table 1). Several co-cultivation parameters were evaluated for their effect on transformation frequencies. One critical factor was the length of time the explants were co-cultivated with *Agrobacterium*. Co-cultivation periods of 24, 48, and 72 h were compared to determine the co-cultivation time that resulted in the greatest transformation efficiency while retaining shoot regeneration capacity. In the absence of selection, the frequency of shoot regeneration was slightly reduced when explants were co-cultivated for 48 and 72 h compared to 24 h (data not shown). However the greatest number of green kanamycin-resistant calli as well as kanamycin-selected, NPT II positive shoots were recovered from explants co-cultivated for 48 h; therefore a 48 h co-cultivation period was used in the standard transformation procedure.

Selection conditions, including kanamycin concentration and the optimal time to begin selection were

Table 1. Shoot transformation frequencies from *Brassica napus* cv. Westar

A. tumefaciens strains	Kanamycin concentration[a] (mg/l)	No. of shoots per total explants (%)	NPTII$^+$ shoots per total shoots tested	Transformation frequency[b] (%)
Control	0	28/124 (22.6)	–	–
	10	0/164 (0)	–	–
	25	0/80 (0)	–	–
	50	0/82 (0)	–	–
EHA101/pCGN767	0	14/98 (14.3)	–	–
	10	9/233 (3.9)	4/9	1.7
	25	4/122 (3.3)	3/4	2.5
	50	3/121 (2.5)	2/2 (1 n.t.)[c]	1.6[d]
A281/pCGN767	0	3/106 (2.8)	–	–
	10	3/238 (1.3)	1/1 (2 n.t.)	0.4[d]
	25	1/120 (0.8)	1/1	0.8
	50	3/118 (2.5)	1/1 (2 n.t.)	0.8[d]

[a] Kanamycin selection was applied immediately or 1 week after the co-cultivation period
[b] Percentage of co-cultivated explants that produced at least one NPTII positive shoot
[c] n.t. = shoots that were not tested for NPT activity
[d] Conservative estimate of transformation frequency based only on shoots that were tested for NPTII activity

determined. Control hypocotyl explants, not exposed to *Agrobacterium* developed slow-growing pale yellow calli and occasionally bleached shoots when cultured on media containing 10, 25, or 50 mg/l kanamycin which was applied immediately or after one week. Light green calli grew from some of the explants plated on all three concentrations of kanamycin when selection was applied after two weeks. Therefore, selection was applied immediately or one week following co-cultivation. When hypocotyl explants were co-cultivated with *Agrobacterium* strain EHA101 containing pCGN767 and cultured on media containing 10, 25, and 50 mg/l kanamycin 1.7, 2.5, and 1.6% of the explants produced transformed shoots respectively (Table 1). Some shoots which regenerated from hypocotyl explants selected on kanamycin-containing media were negative for NPT II activity. The highest frequency of NPT II negative shoots occurred when hypocotyl explants were selected on 10 mg/l kanamycin.

Shoots regenerated from kanamycin-selected hypocotyl calli (Fig. 2) were excised and placed onto hormone-free medium to allow development into normal shoots. The majority of shoots developed roots when placed on root induction medium. However, two out of ten NPT II positive shoots originating from hypocotyl explants co-cultivated with the strain A281 formed only callus at the base of the stem and not roots.

NPT II enzyme assays

Leaves from regenerated shoots were tested for NPT II activity using the dot blot assay. An example of these results is shown in Fig. 3. Leaf extracts in lanes 2, 3, 5, 6, and 8 showed strong expression of the enzyme while leaf extracts in lanes 4 and 11 showed weak expression. Figure 4 shows the result of a NPT II gel assay performed with extracts of leaves from fully-grown transformed plants. NPT II activity was present in leaves from three plants (767-1b, 767-2b a clone of 767-2a, 767-4a) on which Southern analyses were also done. Plants tested using the NPT II gel assay were also positive when tested using the dot blot method. Dot blot assays were therefore used to test kanamycin-resistant shoots for NPT II activity before transfer to root initiation medium. A total of 36 plants obtained from explants co-cultivated with strains EHA101 or A281 containing pCGN767 were shown to be NPT II positive using the dot blot assay.

Molecular analyses

Integration of the T-DNA containing the tagged napin gene and the NPT II selectable marker gene in the *B. napus* genome was determined by Southern analyses. A radiolabeled DHFR DNA probe hybridized to a 3.6 kb fragment in EcoRI digests of DNA from both plants 767-2a and 767-3a and also to a 1.5 kb fragment in BamHI digested DNA (Fig. 5 A) as predicted by the restriction map of pCGN767 (Fig. 1). Hybridization was not detected with either untransformed *B. napus* DNA or with DNA from the NPT II positive plant 767-4a. However, hybridization with radiolabeled probe corresponding to the NPT II gene (Beck et al. 1982) indicated the presence of T-DNA in all three transformed plants (Fig. 5 B). The predicted pattern for the T-DNA with this probe would include an 8.8 kb frag-

Fig. 2. Transformed *Brassica napus*. L. shoot regenerated from a kanamycin-resistant callus derived from a hypocotyl explant. The hypocotyl explant was co-cultivated with *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) harboring a binary plasmid containing the neomycin phosphotransferase II (NPT II) gene and selected on media containing 25 mg/l kanamycin. NPT II enzyme activity was detected in the leaves

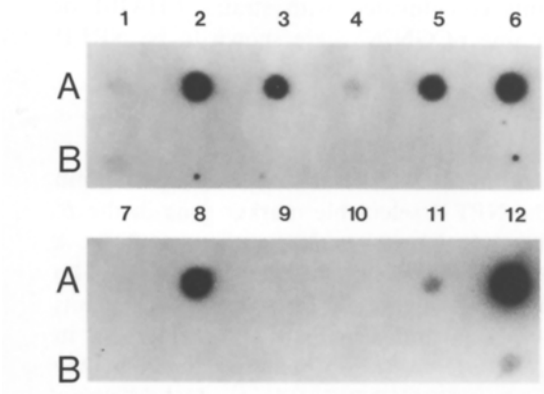

Fig. 3. Neomycin phosphotransferase II enzyme dot blot assay of leaf tissue extracts from *Brassica napus* L. *Row A* represents reactions that contained kanamycin while *row B* represents reactions that did not contain kanamycin. *Lane 1*, untransformed *B. napus* leaf extract. *Lanes 2-11*, leaf extracts from kanamycin-resistant shoots regenerated from hypocotyl explants which were co-cultivated with pCGN767. *Lane 12*, extract from transformed *B. napus* crown gall tissue containing a kanamycin-resistance gene

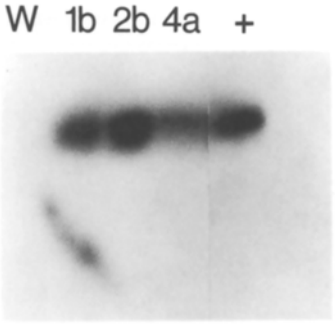

Fig. 4. Neomycin phosphotransferase II (NPT II) enzyme activity in transformed *Brassica napus* L. cv. Westar plants. Leaf extracts were made from an untransformed *B. napus* cv. Westar plant (W), transformed *B. napus* plants pCGN767 *1b, 2b* (clone of plants *2a*), *4a*, and (+) a transformed *B. napus* plant (previously shown to express NPT II activity by the dot blot method), then assayed by the gel electrophoresis method (Reiss et al. 1984)

ment for BamHI digests and variable-sized fragments for EcoRI digests since the probe homology lies in-between an EcoRI site and the right T-DNA border of pCGN767. The multiple-sized EcoRI fragments observed indicated multiple insertions of T-DNAs. Aberrant integration of the T-DNA was evident in transgenic plant 767-4a which contained only one of the two inserted genes. The NPT II gene which was located near the right T-DNA border was integrated (Fig. 5B) but the napin gene with the DHFR fragment was not present (Fig. 5A). The BamHI fragment seen in plant 767-4a (Fig. 5B) is smaller than the expected 8.8 Kb BamHI fragment as seen in plants 767-2a and 767-3a. This suggests that the insertion of the T-DNA was terminated before the second BamHI site from the right border. Three other plants which tested positive for NPT II activity contained no DNA that hybridized with the DHFR probe. However 12 out of 16 of the transgenic plants tested contained both engineered genes.

Transcription of the introduced napin DNA was confirmed by Northern analysis (Fig. 6). A major transcript of 1.2 kb was detected in the RNA from embryos of plants 767-1b and 767-2a and not in the RNA from leaves when probed with a DHFR riboprobe. No DHFR-specific transcript was observed in RNA from either leaves or embryos from a control plant or from plant 767-4a. The expected size of a transcript from the engineered napin gene is approximately 1150 nucleotides, about 300 larger than an endogenous napin message of 850 (Crouch et al. 1983).

Inheritance studies

Germination rates from seeds of transgenic plants were similar to untransformed *B. napus* cv. Westar (> 85%).

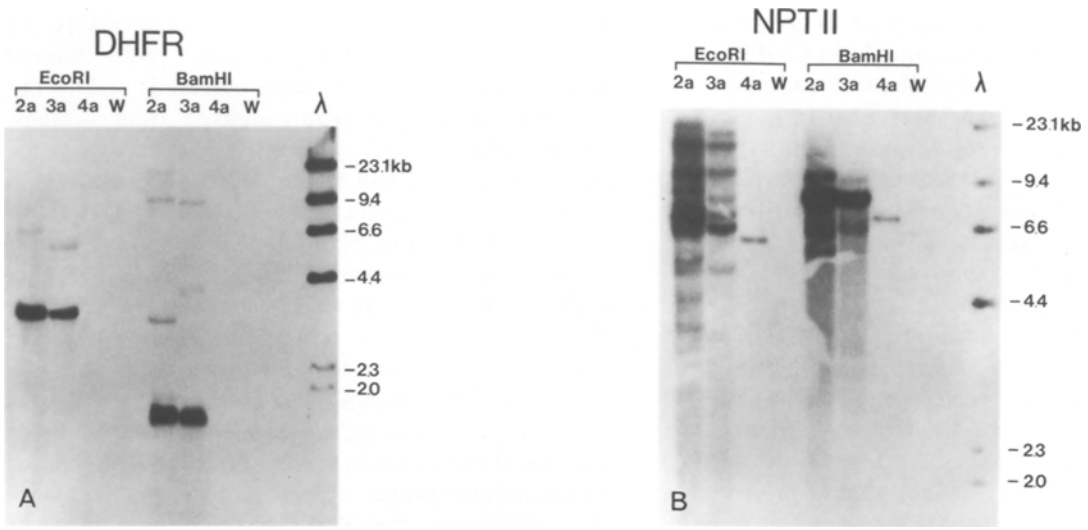

Fig. 5A, B. Southern blot analysis (Southern 1975) of pCGN767-transformed *Brassica napus* L. plants. DNA (10 μg) from leaves of transformed pCGN767 *2a, 3a,* and *4a* plants and an untransformed *B. napus* cv. Westar (W) plant was digested with EcoRI or BamHI restriction enzymes and electrophoresed on 0.7% agarose gels. The gels were blotted to nitrocellulose and probed with ^{32}P-labeled DNA, **A** tagged DHFR fragment as probe or, **B** NPT II DNA fragment as probe. Phage lambda HindIII markers (λ) are shown in kb

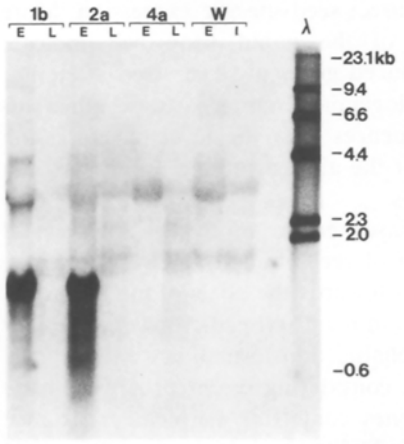

Fig. 6. Northern blot analysis of total RNA from regenerated transformed *Brassica napus* L. plants. Total RNA was isolated from developing embryos (E) and leaves (L) of transformed plants pCGN767-*1b, 2a, 4a* and an untransformed *B. napus* cv. Westar (W) plant and electrophoresed (50 μg/lane) in a denaturing formaldehyde 1.5% agarose gel. The gel was blotted to nitrocellulose and probed with a ^{32}P-labeled RNA transcript corresponding to the DHFR tag DNA

Segregation of the NPT II gene was observed in T_2 generation seeds of plant 767-4a. Sixty-seven out of 88 seedlings were tolerant to kanamycin suggesting a 3 : 1 segregation ratio. This would be expected if the T-DNA integrated into a single locus. All 58 T_2 seedlings derived from 767-1a (clone of 767-1b) and 83 seedlings from 767-2a were tolerant to kanamycin. These data are consistent with hybridization patterns observed in Southern blot analyses of plants 767-1a (data not shown) and 767-2a (Fig. 5 B) suggesting that multple T-DNA integration events into several chromosomal locations occurred. When 27 T_2 plants from 767-1a were rechecked by the NPT II dot blot assay all showed positive enzyme activity as predicted by screening germinating seedlings on kanamycin-containing media.

Discussion

A transformation system using binary disarmed and oncogenic strains of *A. tumefaciens* was developed for *B. napus.* A reintroduced tagged seed storage protein gene with 300 base pairs of its own 5′ region as the promoter and a selectable marker NPT II gene driven by the 35S promoter of CaMV have been expressed in transgenic *B. napus* plants and shown to be stably inherited in progeny. Reports of other explant transformation systems using Ti plasmids in species such as tobacco and petunia (Horsch et al. 1985), tomato (McCormick et al. 1986; Fillatti et al. 1987a), and *Populus* (Fillatti et al. 1987b) have reported much higher transformation rates than we have shown here for *B. napus.* We have attempted *B. napus* transformation using a leaf disc system similar to tobacco and petunia systems (Horsch et al. 1985; Comai et al. 1985) but found lower regeneration frequencies and difficulties in obtaining transformed plants. Efficient shoot regeneration from

B. napus stem explants (Stringham 1977) and thin cell layer explants (Klimaszewska and Keller 1985) have been reported; however non-sterile tissue must be obtained from mature plants grown in specific environmental conditions. Shoot regeneration from hypocotyl explants (Dietert et al. 1982) and hypocotyl protoplasts (Glimelius 1984; Chuong et al. 1985; Barsby et al. 1986) have also been reported. During preparation of this manuscript, a procedure for transformation of *B. napus* stem explant cells was reported (Pua et al. 1987) using methotrexate as a selectable agent. The use of the NPT II gene as a selectable marker was also attempted but did not yield transformants. As described in their approach, 4–5 week old plants were grown in large sterile containers to provide starting material for the transformation process which also involves selection at rooting as well as shooting steps of plant regeneration. The advantages of the hypocotyl explant transformation and regeneration system reported here are the ease in generating uniform sterile seedlings, and in excising and co-cultivating large numbers of hypocotyl explants.

When gene transfer is attempted using explants, it is convenient to have a selection scheme whereby transformed cells can be separated from untransformed cells. The NPT II gene has proven useful as a selectable marker in many plant species (Fraley et al. 1986) and was also effective in our *B. napus* transformation system. Transformed calli and shoots remained green while untransformed tissues bleached on kanamycin-containing medium. Some green shoots regenerated from kanamycin-selected calli subsequently tested negative for NPT II activity while other shoots originating from the same explant expressed NPT II activity. This has been observed in other species (tomato, tobacco, petunia) as well, however in these species shoots are selected a second time on root induction medium containing kanamycin to further reduce the frequencies of escapes. We have attempted selection of *B. napus* transformants on kanamycin-containing root induction medium. As expected NPT II positive shoots remained green and most developed roots whereas NPT II negative shoots bleached. However some NPT II negative shoots developed roots suggesting root formation is not blocked by the presence of kanamycin.

The advantage of the disarmed *A. tumefaciens* system is that transgenic *B. napus* plants with a normal phenotype and normal seed set were obtained. The major drawback to using *A. rhizogenes* as a gene vector in *Brassica* is that regenerated transgenic plants generally exhibit altered morphologies including wrinkled leaves and varying degrees of sterility (Oom et al. 1985; Guerche et al. 1987). We observed some transgenic plants with altered floral structure using our disarmed *A. tumefaciens* transformation system which resulted in poor seed set. The most common abnormality we observed was flowers with elongated pistils. Similar morphological alterations were observed, at low frequencies, in *B. napus* plants derived from untransformed cells cultured *in vitro* (D. Facciotti, personal communication). These abnormalities were usually not observed in subsequent generations. Despite the occurrence of abnormal plants we can use the disarmed *A. tumefaciens* transformation system to successfully regenerate transgenic *B. napus* plants with normal morphologies on a routine basis. Seeds can be collected from transgenic plants 6–7 months after explant cocultivation with *A. tumefaciens.*

The expression of the tagged napin gene in embryos and lack of expression in leaves of transformed plants is consistent with proper expression of the native napin gene. Moreover, the 300 nucleotide promoter was sufficient to direct expression of the tagged gene in embryos but not in leaves. Other storage proteins of bean and soybean have also been shown to express in a seed-specific manner when transformed into heterologous hosts (Sengupta-Gopalan et al. 1985; Beachy et al. 1985). When soybean β-conglycinin was transferred to petunia only 257 nucleotides of 5′ upstream region were required to direct seed-specific expression. More detailed analyses of the napin gene will indicate whether the introduced genes are expressed at similar levels as their endogenous counterparts, whether additional DNA sequences alter the level of expression and whether or not the altered protein accumulates in developing embryos.

We have demonstrated the ability to transfer and regulate expression of recombinant DNA in *B. napus.* These are key steps towards the goals of modifying oil and protein content in major oilseed crops. Availability of a convenient, reliable transformation system for *B. napus* is significant considering recent progress on the development of genes conferring herbicide resistance (Comai et al. 1985; Stalker and McBride 1987), viral resistance (Beachy et al. 1985) and insect resistance (Vaeck et al. 1987). Given the relatedness of *Brassica* species, we predict that the hypocotyl explant cocultivation system described here can also be extended to important vegetable crops included in the species *B. oleracea* and *B. campestris.* We have recently extended these results with the spring variety *B. napus* cv. Westar to include other seed-specific gene constructs introduced into winter varieties of *B. napus.*

Acknowledgements. The authors would like to thank David McCarter for technical assistance in Southern and Northern analyses, David Shintani for technical assistance in the construction of the binary plasmid, and Cleofe Lastrella and Dauphine Torrance for their technical assistance. We also thank Steve Scofield and Keith Blundy for their helpful discussions and Daniel Facciotti, JoAnne Fillatti and Robert Goodman for their critical review of the manuscript.

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