Theor Appl Genet (1988) 75:438–445

Agrobacterium-mediated transformation of thin cell layer explants from *Brassica napus* L.*

P. J. Charest[1], L. A. Holbrook[2], J. Gabard[2], V. N. Iyer[1] and B. L. Miki[2]*****

[1] Department of Biology, Carleton University, Ottawa, Ontario K1S 5B6, Canada
[2] Plant Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada

Received May 29, 1987; Accepted September 21, 1987
Communicated by P. Maliga

Summary. *Agrobacterium*-mediated transformation of thin cell layer explants (Klimaszewska and Keller 1985) yielded large numbers of transgenic plants of a major Canadian rapeseed cultivar *Brassica napus* ssp. *oleifera* cv Westar. The morphology and fertility of these plants were indistinguishable from controls. The Ti plasmid vector, pGV3850 (Zambryski et al. 1983) was used as a cis vector and as a helper plasmid for the binary vector pBin19 (Bevan 1984). Selectable marker genes that conferred resistance to high levels of kanamycin (Km) on *Nicotiana tabacum* were less efficient in the selection of transgenic *B. napus.* At low levels of Km (15 μg/ml) large numbers of transgenic plants (50%) were identified among the regenerants by nopaline synthase activity and several of these were confirmed by Southern blot analyses. Only a small number were resistant to higher levels of Km (80 μg/ml). Preliminary analyses indicated that resistance to Km was transmitted to the selfed progeny. Chimeric chloramphenicol acetyl transferase genes were ineffective biochemical markers in transgenic *B. napus.*

Key words: *Brassica napus* – Rapeseed – Transformation – *Agrobacterium tumefaciens* – Ti plasmid

Introduction

The genus *Brassica* encompasses a wide range of economically-important crop species including rapeseed. Recent advances in the cell and tissue culture technologies for rapeseed have enabled the genetic manipulation of the cytoplasmic genomes (Pelletier et al. 1983; Yarrow et al. 1986). Continued advances using genetically-engineered nuclear genes depends on the development of methods for producing transgenic plants. *Brassica* species in general are excellent hosts for transformation by Ti and Ri plasmids (Holbrook and Miki 1985; Mathews et al. 1985, 1986; Tanaka et al. 1985; Ooms et al. 1985). Recently, fertile, transgenic *B. napus* have been generated with the Ri plasmid of *A. rhizogenes;* however, these plants expressed the morphological abnormalities and reduced fertility imposed by the Ri T DNA (Guerche et al. 1987).

In this report, the transformation of a major Canadian rapeseed cultivar, *B. napus* ssp. *oleifera* cv. Westar with *A. tumefaciens* strains harboring disarmed Ti plasmid vectors is demonstrated. Thin cell layer explants which yielded consistently high frequencies of plant regeneration with cultivar Westar (Klimaszewska and Keller 1985), were adopted as hosts for transformation. The major obstacle in the regeneration of transgenic plants was the hypersensitive response of the explants to the *A. tumefaciens* strains. Once conditions were determined that overcame this problem, transgenic plants that were indistinguishable from controls were recovered.

Materials and methods

Plants, cultures and strains

Seeds of *B. napus* ssp. *oleifera* cv Westar were supplied by R. K. Downey, Agriculture Canada (Saskatoon, Canada). *Brassica napus,* Jet Neuf, cell suspension cultures were obtained from W. Keller, Agriculture Canada (Ottawa, Canada).

* Contribution No. 1092 Plant Research Centre, Ontario, Canada
** Present address: Biotechnica International of Canada Inc., 170-6815 8 Street NE, Calgary, Alberta T2E 7H7, Canada
*** To whom correspondence should be addressed

The *A. tumefaciens* strains used in this study are described in Table 1. The chimeric genes *nos-nptII-ocs* (5'region-coding sequence-3' eno), *nos-CAT-nos, 35S-CAT-nos* (Hain et al. 1985; DeBlock et al. 1984; M. Bevan, unpublished) were evaluated as selectable markers. The *nos-CAT-nos* and *nos-nptII-ocs* genes were cointegrated with the T DNA of pGV3850 using the intermediate vectors pNCAT4 (DeBlock et al. 1984) and pLGV1103neo (Hain et al. 1985) to form the two cis vectors pGV3850: :pNCAT4 and pGV3850: : pLGV1103 neo. The *35S-CAT-nos* gene was inserted into the binary vector pBin19 (Bevan 1984) by M. Bevan to form pBCATI (unpublished) and this vector was maintained in *A. tumefaciens* with pGV3850 acting as the helper plasmid. Vector pBCATI also carried a chimeric *nos-nptII-nos* gene.

Transformation of thin cell layers

Thin cell layer explants used in cocultivation studies with *A. tumefaciens* were prepared according to the specific conditions determined by Klimaszewska and Keller (1985). Modifications to the tissue culture procedures were made to compensate for the use of the explants in cocultivation experiments. Prior to inoculation with bacteria, *B. napus* explants were preconditioned by culture over a feeder layer of *B. napus,* Jet Neuf, cells from suspension cultures using the double filter paper technique (Horsch and Jones 1980). After one to four days, explants were dipped in an overnight bacterial culture grown in LB medium and diluted with distilled sterile H_2O or media. The explants were blotted on filter paper and cocultivated for one to three days. To inhibit bacterial growth, the explants were transferred to media solidified in 0.7% agarose (Seakem ME, FMC) to which 0.5 mg/ml carbenicillin (Ayerst Labs, Montreal, Canada), 0.5 mg/ml Clavulin 250 (Beecham Labs Inc., Montreal, Canada), 0.5 mg/ml cefotaxime (Terochem Lab. Ltd., Mississauga, Canada) or 0.5 mg/ml Claforan (Roussel Canada, Montreal, Canada) was added.

Selection conditions

At various times, the explants were transferred to media that also contained 15 μg/ml kanamycin (Km) or 7 μg/ml chloramphenicol (Cm) and subcultured approximately every two weeks. Well-developed shoots were transferred to B_5 media lacking growth regulators to induce roots in the presence of Km or Cm and plantlets were transferred to peat pellets prior to soil (Klimaszewska and Keller 1985).

The effect of Km and Cm on growth and differentiation was examined. Explants were transformed with an *A. tumefaciens* strain carrying only pGV3850 (GV3101, Table 1) and cultured on media at six different antibiotic concentrations. A minimum of 12 explants were cultured at each concentration. The fresh weights measured after five weeks and the average fresh weight was expressed as a percentage of the average fresh weight of tissues growing in the absence of Km or Cm.

Opine synthase analyses

Nopaline synthase and octopine synthase activities were analyzed by precursor labelling of nopaline or octopine with [^{14}C]-arginine, followed by paper electrophoresis and fluorography (Holbrook et al. 1986). For each plant examined, approximately 10 randomly selected tissue samples were analyzed.

Southern blot analyses

Plant DNA was isolated from leaves by methods described by Lichtenstein and Draper (1985). Southern blot analyses were performed according to Bernatsky and Tanksley (1986).

Inheritance of antibiotic resistance

Regenerated plants were self-pollinated. The seeds were sterilized with 0.2% (w/v) mercuric chloride, 20–25 min and 6% (w/v) sodium hypochlorite 20–30 min. The seeds were rinsed in sterile distilled water and placed on MS medium with B_5 vitamins, 1.0% sucrose, 0.6% agarose. Resistance or sensitivity to 20 μg/ml Km or 40 μg/ml Cm was scored after five to six weeks.

Results

Transformation of thin cell layer explants

Brassica napus thin cell layer explants (Klimaszewska and Keller 1985) were evaluated as hosts for *Agrobacterium*-mediated transformation. The uninoculated explants were highly regenerable in culture producing on average, five shoots per explant within three to four weeks. Exposure to *A. tumefaciens* strains following the

Table 1. *Agrobacterium tumefaciens* strains and plasmids used for the transformation of *Brassica napus*

Strain	Chromosomal background	Ti plasmid[a]	Intermediate vector[b]	Binary vector[c]	Marker genes
1. C58	C58	pTiC58	–	–	*nos* (aut)[d]
2. GV3101	C58	pGV3850	–	–	*nos*
3.			pNCAT4	–	*nos, nos-CAT-nos*
4.			pLGV1103neo	–	*nos, nos-nptII-ocs*
5.				pAL1050	*ocs,* (aut)[d], (*nos*)[e]
6.			–	pBCAT1	*nos-nptII-nos, 35S-CAT-nos,* (*nos*)[e]
7. B6S3	B6S3	pTiB6S3	–	–	*ocs,* (aut)[d]

[a] pTiC58 (Holsters et al. 1980); pGV3850 (Zambryski et al. 1983); pTiB6S3 (Petit and Tempe 1978)
[b] pNCAT4 (DeBlock et al. 1984); pLGV1103neo (Hain et al. 1985)
[c] pAL1050 (Hoekema et al. 1983); pBCATI (M. Bevan, unpublished)
[d] (aut). In culture, growth in the absence of growth regulators was used as a selectable marker for T-DNA of natural Ti plasmids
[e] (*nos*). In strains harbouring binary vectors, nopaline synthase activity was used as a marker for transformation by T-DNA of pGV3850

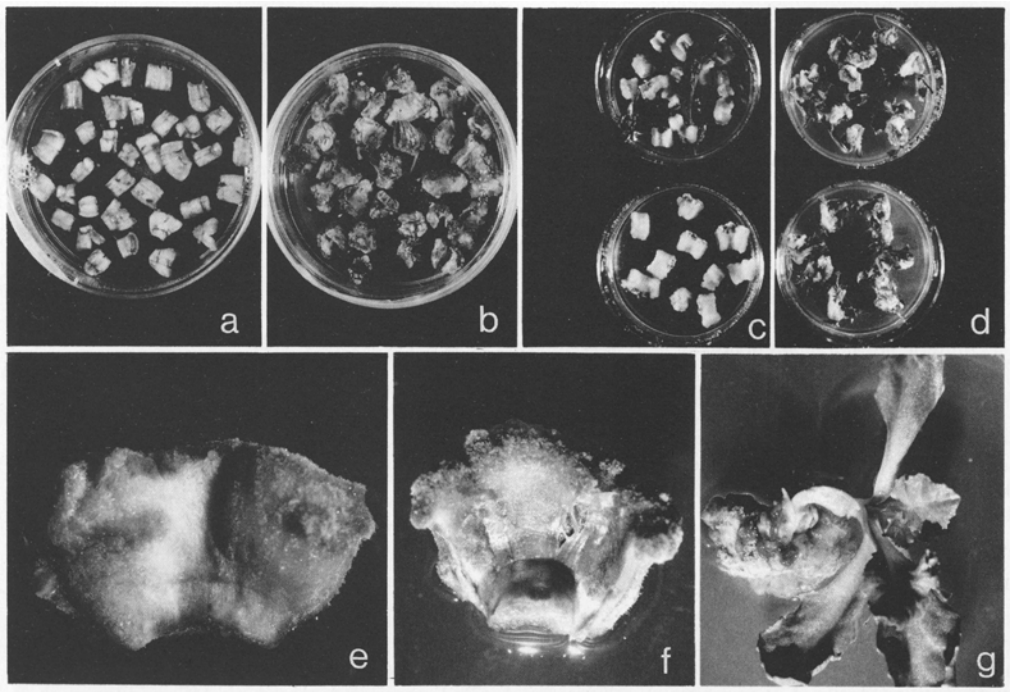

Fig. 1a–g. Thin cell layer explants cocultivated with *A. tumefaciens* strain GV3101. **a, e** Explants prepared according to Klimaszewska and Keller (1985) and cocultivated using the leaf disc transformation procedure (Horsch et al. 1985) were chlorotic and brown after three weeks. **b, f** Viable explants with green meristematic regions after three weeks were recovered using the modified conditions for inoculation and culture. **d, g** Shoot differentiation was achieved by six weeks. **c** The frequency of shoot differentiation was reduced on media solidified in 0.8% agar

Table 2. Transformation rates of thin cell layer explants of *B. napus*[a]

Strain	Plasmids	Precondition-cocultivation[b]		
		0-2	4-2	4-1
C58	pTiC58	50	100	16
B6S3	pTiB6S3	0	48	0
GV3101	pGV3850, pAL1050	N.T.	79	N.T.

[a] Transformation rates were measured as the percentage of infected explants that demonstrated nopaline synthase (pTiC58) or octopine synthase (pTiB6S3, pAL1050) activities. Measurements were based on the analyses of at least 15 explants

[b] The explants were cocultivated with the *A. tumefaciens* strains for two days without preconditioning (0-2) of after preconditioning for four days (4-2). Explants were also cocultivated for 1 day after preconditioning for four days (4-1)

N.T. = not tested

procedure for leaf disc transformation (Horsch et al. 1985) induced a severe hypersensitive response that completely prohibited the recovery of viable tissues (Fig. 1 a, e). The procedures for inoculation and culture of explants were therefore modified to minimize tissue necrosis (Fig. 1 b, d, f, g) and to enhance explant transformation rates (Table 2).

The inoculation of the explants with the bacterial strains was a critical step in the recovery of healthy tissues. With suspensions of 10^9 bacteria per ml, inoculations could not exceed 30s because rapid bacterial growth during cocultivation could not be inhibited by carbenicillin, cefotaxime, Claforan or Clavulin 250 at 500 µg/ml. Shorter inoculations permitted cocultivation periods up to two days and subsequent inhibition of bacterial growth by carbenicillin or Clavulin 250. Cefotaxime and Claforan were toxic to the explants under these conditions of culture. In the recovered tissues necrosis was still evident and the frequency of shoot differentiation was suppressed to about 0.6 shoots per explant (Fig. 1 c).

A dramatic reversal of tissue necrosis was achieved by replacing agar in the media with 0.7% agarose (Fig. 1 b, d, f, g). Shoot differentiation was restored to levels of three to five shoots per explant (Fig. 1 d, g); however, this was delayed by one to two weeks relative to uninoculated controls. Preconditioning of explants prior to inoculation with feeder layers of *B. napus* cell suspension cultures did not alter these results significantly but appeared to be associated with greater consistency among experiments in the recovery of viable tissues. Under these improved culture conditions, any of the antibiotics carbenicillin, cefotaxime,

Claforan or Clavulin 250 were effective in the control of bacterial growth without detriment to plant tissue growth and differentiation.

Explant transformation rates were assessed by analyses of opine synthase activities in tissues transformed with pTiC58 or pTiB6S3 and selected on media lacking growth regulators. With both strains, a four-day preconditioning period and a two-day cocultivation were effective (Table 2). Longer durations of preconditioning had not been examined. Longer cocultivation resulted in uninhibited bacterial growth. Transformation rates for pTiC58 were consistently high and for pTiB6S3 were always much lower (Table 2). The same conditions applied to a strain harboring the disarmed Ti plasmid vector pGV3850 and the binary T-DNA vector pAL1050 yielded high transformation rates for pAL1050 (Table 2). A large proportion (68%) of these were also transformed with pGV3850 revealed by the presence of both octopine synthase and nopaline synthase activities.

Selection and regeneration of transformants

The effect of the antibiotics Km and Cm on the growth and differentiation of thin cell layer explants is illustrated in Fig. 2. These profiles were determined with explants after cocultivation with a strain carrying only pGV3850 (GV3101, Table 1). At 35 μg/ml Km, shoot differentiation was completely abolished and tissue growth was significantly retarded (Fig. 2). Following cocultivation with strains carrying pGV3850: : pLGV1103 neo or pGV3850 and pBCATl, shoots did not differentiate in the presence of 35 μg/ml Km despite repeated attempts. The same strains were used to recover tobacco shoots resistant to 100 μg/ml Km (data not shown). At 15 μg/ml Km many shoots differentiated but several were gradually lost at later stages of growth and differentiation (Fig. 3 a, b). Typically, these shoots became chlorotic and would not form roots before dying (Fig. 3 b). Many that survived and formed roots in the presence of 15 μg/ml Km after one to two months (Fig. 2 b) were successfully transferred to soil after two to three months. Approximately one-half of the plants displayed nopaline synthase activity following transformation with pGV3850: : pLGV1103 neo. Most of them displayed activity after transformation with pGV3850 and pBCATl; however, nopaline synthase activity in these plants reflected transformation by the T-DNA of pGV3850 whereas the *nos-nptII-nos* and 35S-CAT-*nos* genes are carried by pBCATl. A summary of these experiments is presented in Table 3.

Chloramphenicol at levels above 20 μg/ml inhibited explant growth and differentiation; however, resistant shoots could not be recovered at these levels after

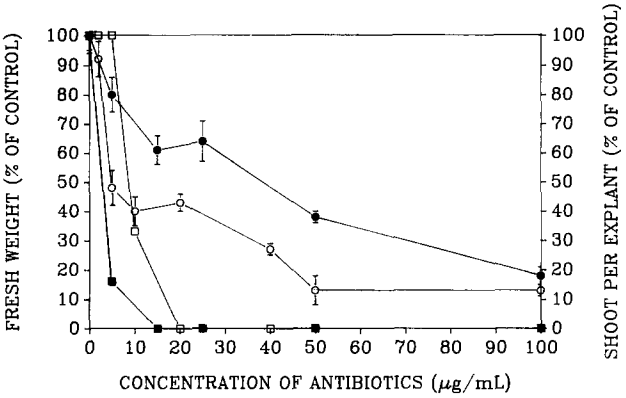

Fig. 2. Influence of Km and Cm on the growth and differentiation of thin cell layer explants. At least 12 explants (fresh weight 10±2 mg) cocultivated with strain GV3101 were incubated on media with antibiotics at each of the concentrations indicated (Km ●–●, ■–■; Cm ○–○, □–□). The average fresh weight after five weeks (●–●, ○–○) were expressed as a percentage of the average fresh weight of explants incubated without antibiotics (601±32 mg). Bars indicate standard errors. The frequency of shoot differentiation (■–■, □–□) was expressed as a percentage of the frequency (shoots per explant) measured for explants incubated without antibiotics

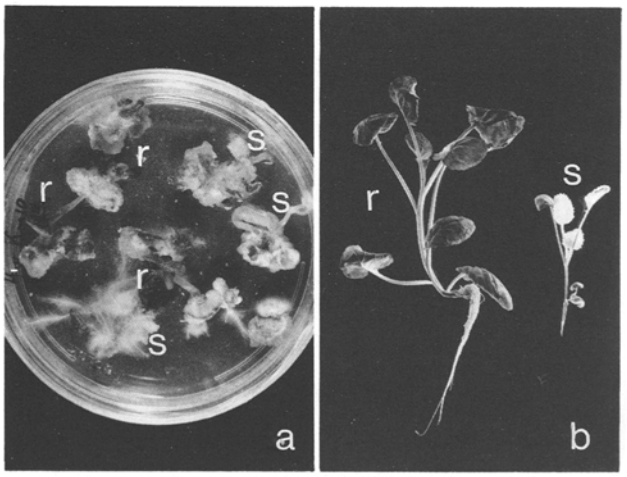

Fig. 3 a, b. Selection of transformed shoots and plantlets on Km. Thin cell layer explants were cocultivated with *A. tumefaciens* harbouring pGV3850::pLGV1103neo and cultured on 15 μg/ml Km. **a** Shoot differentiation occurred in the presence of Km; however, many shoots became chlorotic (s) and died. **b** The survivors (r) were excised and roots were induced in the presence of Km. Many of them did not form roots. They became chlorotic and eventually died (s). Others formed roots in the presence of Km (r), and were transferred to soil

transformation with pGV3850: :pNCAT4 or pBCATl. Reduction of Cm to 7 μg/ml yielded many shoots but tissues transformed with pGV3850 alone also yielded shoots at this level despite a modest suppression of tissue growth (Fig. 2). About one half of the plants recovered following tansformation by pGV3850: :

Table 3. Recovery of transformed *B. napus* plants

Plasmids	Selection[a]	Explants inoculated	Resistant shoots[b]	Plantlets in soil	Nopaline synthase[d]
pGV3850:: pLGV1103neo	Km	600	40/80	26	12
pGV3850::pNCAT4	Cm	600	30/70	19	10
pGV3850, pBCAT1[c]	Km, Cm	200	10/40	6	5

[a] Antibiotic selections were performed at 15 μg/mL Km or 7 μg/mL Cm

[b] The resistant shoots are indicated as a proportion of the total shoots that differentiate in the presence of low levels of antibiotics

[c] Explants transformed with pBCAT1 were subjected to selection on Km or Cm. Each of the selections yielded three plants. These were analyzed for nopaline synthase activity which was encoded by pGV3850

[d] Nopaline synthase activity was determined for plants established in soil. At least 10 samples were taken from each plant for analyses because of variability that existed within inidividual plants

Table 4. Inheritance of Km^r in self-pollinated transgenic *B. napus* plants

Plasmids	Plant	$Km^r : Km^{s,a}$
pGV3850::pLGV1103neo	NEO4A	9:6
	NEO12A	8:5
	NEO8B	7:4
	NEO12B	12:4
	Control	0:25
pGV3850, pBCAT1	BCAT1B	7:8
	Control	0:25

[a] Seedlings were germinated in media with 20 μg/ml Km and resistance was scored after five to six weeks

pNCAT4 were associated with nopaline synthase activity (Table 3).

Several of the plants potentially transformed with pBCAT1 displayed nopaline synthase activity encoded by pGV3850 (Table 3). CAT activity as a direct biochemical marker for transformation was impossible to assess because all of the tissues were associated with very high levels of endogenous CAT activity (data not shown).

Analyses of transformants

All of the plants that displayed nopaline synthase activity flowered and set seed within five to six months. In morphology and fertility, these plants were indistinguishable from control plants derived from seeds and grown under identical conditions (Fig. 4).

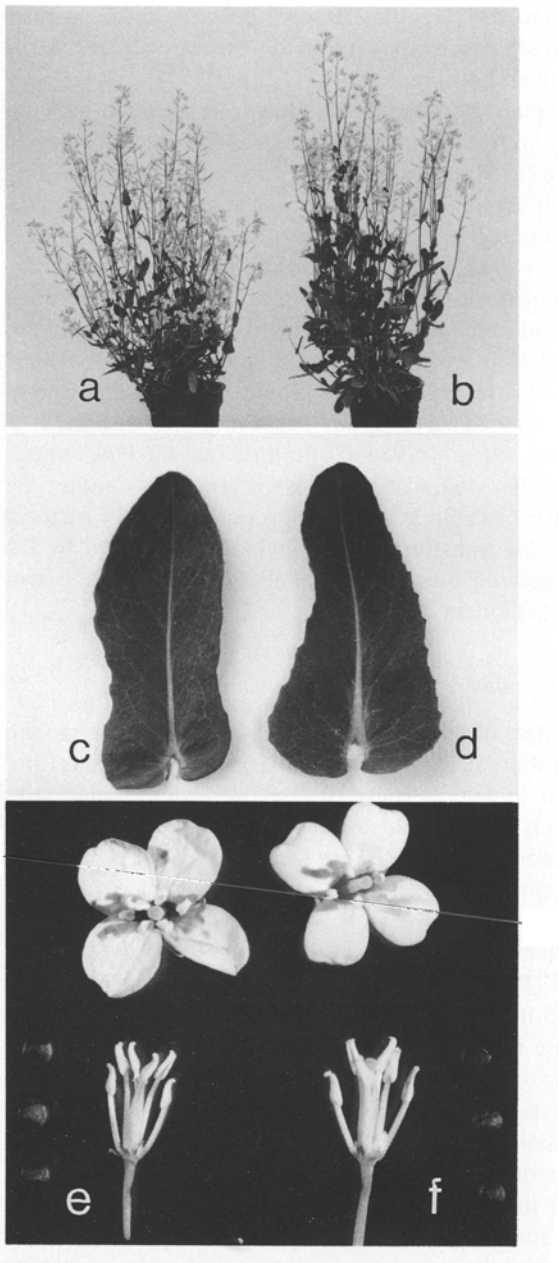

Fig. 4. Comparison of regenerated transgenic plants with normal *B. napus* cv. Westar plants. **a** Transgenic plant NEO8B transformed with pGV3850::pLGV1103neo. **c, e** leaf; flower, floral parts, seeds derived from NEO8B. **b** Normal *B. napus* cv. Westar plants grown from seed. **d, f** Leaf; flower, floral parts, seeds derived from normal plants

Southern blot analyses confirmed the integration of vector DNA at the right T-DNA border in six out of seven plants tested. With a fragment of the *nos* gene as the hybridization probe, two separate integration events were revealed for a representative plant (NEO12B) transformed with pGV3850: :pLGV1103 neo and another representative plant (NCAT7B) transformed with

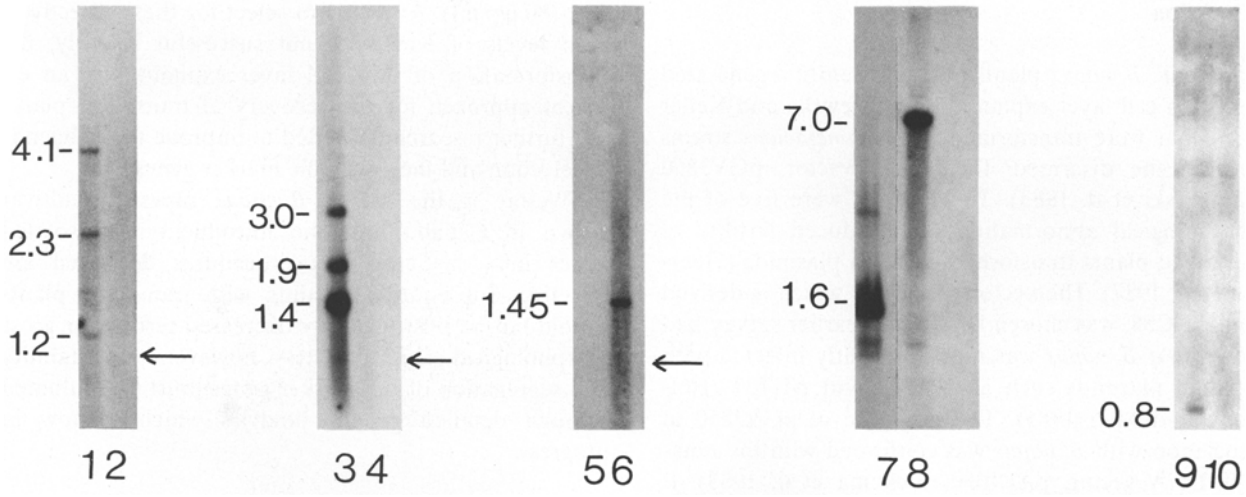

Fig. 5. Southern blot analyses of transgenic plants. The arrows indicate the minimum fragment size that would encompass the T DNA-plant DNA junction at the right border of pGV3850 following digestion with BamHI and HindIII and hybridization with the BamHI-SstII fragment from the HindIII 23 fragment of pTiC58 as the probe. DNA from transgenic plant NCAT7B transformed with pGV3850::pNCAT4 was digested with BamHI and HindIII (*lane 1*). The 1.2 kb BamHI-HindIII fragment of pNCAT4 is indicated. Two T DNA-plant junction fragments of 4.1 kb and 2.3 kb were revealed. DNA from transgenic plant NEO12B transformed with pGV3850::pLGV1103neo was digested with PstI, BamHI and HindIII (*lane 3*). Two T DNA-plant DNA junction fragments of 3.0 kb and 1.9 kb were revealed in addition to the 1.4 kb PstI fragment of pLGV1103neo. DNA from transgenic plant BCAT1B transformed with pGV3850, pBCAT1 was digested with BamHI and HindIII (*lane 6*). A single T DNA-plant DNA junction fragment of 1.45 kb was revealed. Comparable bands were not found in DNA from untransformed control plants (*lanes 2, 4, 5*). The 1.6 kb PstI fragment of pLGV1103neo was used as the probe to reveal the same fragment which contains the coding region of *nptII* in the DNA of NEO12B digested with PstI (*lane 7*). The coding region of *nptII* was also demonstrated in a 7.0 kb fragment from DNA of transgenic plant BCAT1B digested with PstI (*lane 8*). The 0.8 kb BamHI fragment of pNCAT4 was used as the probe to reveal the same fragment which contains the coding region of chloramphenicol acetyl transferase gene in the DNA of NCAT7B digested with BamHI (*lane 9*). A comparable band is not found in DNA from untransformed control plant (*lane 10*). Ten μg DNA were loaded into each lane

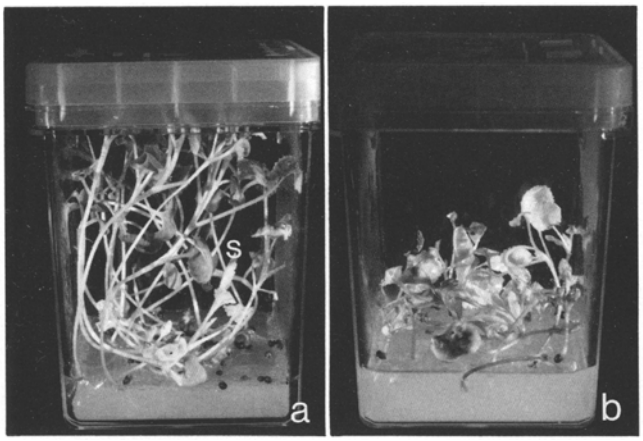

Fig. 6a, b. Segregation of Km^s and Km^r seedlings. **a** Seeds from a self-pollinated transgenic plant NEO12B and **b** control *B. napus* plants were germinated in the presence of 20 μg/ml Km. Sensitive seedlings (s) were chlorotic and growth was inhibited. Resistant seedlings were green and displayed normal growth characteristics

pGV3850: :pNCAT4 (Fig. 5). A single integration event is shown for another plant (BCAT1B) transformed with a strain carrying pBCAT1 and pGV3850 (Fig. 5). The presence of the coding region of the *nptII* gene was confirmed in both NEO12B and BCAT1B and the coding region of the *CAT* gene was confirmed in NCAT7B (Fig. 5).

To assess the level of antibiotics that could be tolerated, shoots were cloned from the axillary buds of plants selected on 15 μg/ml Km that displayed nopaline synthase activity encoded by pGV3850: : pLGV1103 neo. Shoots from each were subcultured on media with 20, 40, or 80 μg/ml Km. In one transformation experiment, 13 plants were sensitive to 20 μg/ml Km. One plant was tolerant to 40 μg/ml Km but not 80 μg/ml. Another plant was resistant to all of the levels tested. Similar experiments, with eight plants transformed with pGV3850: :pNCAT4 were subjected to various levels of Cm; however, the results were inconclusive due to the inherent and variable tolerance of differentiated *B. napus* shoots to this antibiotic.

Preliminary genetic analyses were performed on a limited number of plants. Seedlings from control plants that germinated in the presence of 20 μg/ml Km were uniformly chlorotic and severely inhibited in growth (Fig. 6 b). Among the progeny of self-pollinated plants that were transformed with pGV3850: :pLGV1103 neo or pBCAT1 resistance to 20 μg/ml was evident (Fig. 6 a).

Discussion

Transgenic *B. napus* plants were efficiently regenerated from thin cell layer explants (Klimaszewska and Keller 1985) that were transformed by *A. tumefaciens* strains carrying the disarmed Ti plasmid vector, pGV3850 (Zambryski et al. 1983). These plants were free of the morphological abnormalities and reduced fertility of transgenic plants transformed with Ri plasmids (Guerche et al. 1987). The vector pGV3850, which is derived from pTiC58, was chosen because an earlier survey had shown that *B. napus* was most efficiently infected with nopaline plasmids such as pTiC58 and pTiT37 (Holbrook and Miki 1985). The virulence of pGV3850 in association with *B. napus* was confirmed with the binary T DNA vector, pAL1050(Hoekema et al. 1983). It was employed effectively both as a cis vector and as a helper plasmid for the binary vector pBin19 (Bevan 1984).

A survey of methods described for the regeneration of *Brassica* plants from tissue explants (Kartha et al. 1974; Margara and Leydecker 1978; Stringam 1979; Dunwell 1981; Lazzeri and Dunwell 1984a, b; Klimaszewska and Keller 1985) revealed that thin cell layer explants (Klimaszewska and Keller 1985) were particularly regenerable and well suited for the experimental manipulations involved in transformation by *A. tumefaciens.* A major obstacle was the severe hypersensitive response of the tissues to the *A. tumefaciens* strains and the associated loss of morphogenesis. This problem was overcome by determining the optimal conditions for inoculating the explants and by modifying the culture conditions to enhance tissue survival.

Large numbers of transgenic plants have been produced with the methods described in this study. Nopaline synthase activity (Holbrook et al. 1986) was a reliable biochemical marker for identifying them. Chloramphenicol acetyl transferase activity could not be used because of the high levels of endogenous activity found in all of the tissues examined. A similar finding has recently been reported (Balazs and Bonneville 1987). Furthermore, chloramphenicol was a poor selective agent for differentiated shoots and seedlings because of the inherent and variable tolerance of these tissues to this antibiotic. Despite this, a large number of plants transformed with chimeric *CAT* genes have been recovered and verified by Southern blot analyses. As selectable marker genes *nos-nptII-nos* (Bevan 1984) and *nos-nptII*-ocs (Hain et al. 1985) did not provide selection of transformed *B. napus* shoots that could differentiate fully in the presence of high levels of Km; however, gradual selection at low levels of Km (15 μg/ml) yielded a high proportion of transformants among the regenerated plants (50%). Only a small number of these were also resistant to high levels of Km (80 μg/ml). Attempts to select for these directly at high levels of Km were not successful. Clearly, the transformation of thin cell layer explants was an efficient approach for the recovery of transgenic plants but further research is needed to improve the efficiency of selection and the selectable marker genes.

Westar is the major *B. napus* rapeseed cultivar grown in Canada. For the introduction of isolated genes into this crop, the procedures described are effective. Subsequent breeding with transgenic plants should not be obstructed by decreased fertility or gross morphological abnormalities; however, the stability and segregation of the marker genes must be evaluated through detailed genetic analysis which is now in progress.

Acknowledgements. P. Charest is the recipient of NSERC and FCAR post-graduate scholarships. L. Holbrook is the recipient of an NSERC Visiting Fellowship. J. Gabard is on leave from INRA. Plant Research Centre publication number 1092.

References

Balazs E, Bonneville JM (1987) Chloramphenicol acetyl transferase activity in *Brassica* spp. Plant Sci 50:65–68

Bernatzky R, Tanksley SD (1986) Methods for detection of single or low copy sequences in tomato on Southern Blots. Plant Mol Biol Rep 4:37–91

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 12:8711–8721

De Block M, Herrera-Estrella L, Van Montagu M, Schell J, Zambryski P (1984) Expression of foreign genes in regenerated plants and in their progeny. EMBO J 3:1681–1689

Dunwell JM (1981) In vitro regeneration from excised leaf discs of three *Brassica* species. J Exp Bot 32:784–789

Guerche P, Jouanin L, Tepfer D, Pelletier G (1987) Genetic transformation of oilseed rape (*Brassica napus*) by the Ri T-DNA of *Agrobacterium rhizogenes* and analysis of inheritance of the transformed phenotype. Mol Gen Genet 206:382–386

Hain R, Stabel P, Czernilofsky AP, Steinbiss HH, Herrera-Estrella L, Schell J (1985) Uptake, integration, expression and genetic transmission of a selectable chimeric gene by plant protoplasts. Mol Gen Genet 199:161–168

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of VIR- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179–180

Holbrook LA, Miki BL (1985) *Brassica* crown gall tumourigenesis and *in vitro* culture of transformed tissue. Plant Cell Rep 4:329–332

Holbrook LA, Haffner M, Miki BL (1986) A sensitive fluorographic method for the detection of nopaline and octopine synthase activities in *Brassica* crown-gall tissues. Biochem Cell Biol 64:126–132

Holsters M, Silva B, VanVliet F, Genetello C, De Block M, Dhaese P, Depicker A, Inzé D, Engler G, Villaroel R, Van Montagu M, Schell J (1980) The functional organization of the nopaline, *Agrobacterium tumefaciens* pTiC58. Plasmid 3:212–230

Horsch RB, Jones GE (1980) A double filter paper technique for plating cultured plant cells. In Vitro 16:103–108

Horsch RB, Fry JE, Hoffmann NL, Eicholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227:1229–1231

Kartha KK, Gamborg OL, Constabel F (1974) *In vitro* plant formation from stem explants of rape (*Brassica napus* cv Zephyr). Physiol Plant 31:217–220

Klimaszewska K, Keller WA (1985) High frequency plant regeneration from thin cell layer explants of *Brassica napus*. Plant Cell Tiss and Organ Cult 4:183–197

Lazzeri PA, Dunwell JM (1984a) *In vitro* shoot regeneration from seedling root segments of *Brassica oleracea* and *Brassica napus* cultivars. Ann Bot 54:341–350

Lazzeri PA, Dunwell JM (1984b) Establishment of isolated root cultures of *Brassica* species and regeneration from cultured root segments of *Brassica oleracea* var. *italica*. Ann Bot 54:351–361

Lichtenstein C, Draper J (1985) Genetic engineering of plants in DM Glover; DNA cloning, vol II. IRL Press, pp 67–120

Margara J, Leydecker M (1978) Différents types d'organogénèse observés chez le colza, *Brassica napus* L var. *oleifera* Metzg. Applications à la multiplication végétative *in vitro*. CR Acad Sci Paris 287:17–20

Mathews H, Bhatia CR, Mitra R, Krishna TG, Rao PS (1985) Regeneration of shoots from *Brassica juncea* (Linn) czern and coss cells transformed by *Agrobacterium tumefaciens* and expression of nopaline dehydrogenase genes. Plant Sci 39:49–54

Mathews H, Rao PS, Bhatia CR (1986) Transformation of *Brassica juncea* by *Agrobacterium tumefaciens* harbouring plasmid pTiT37 and its rooty mutant pTiT37.14a/a. J Genet 65:37–44

Ooms G, Bains A, Burrell M, Karp A, Twell D, Wilcox E (1985) Genetic manipulation in cultivars of oilseed rape (*Brassica napus*) using *Agrobacterium*. Theor Appl Genet 71:325–329

Pelletier G, Primard C, Vedel F, Chetrit P, Remy R, Rouselle P, Renard M (1983) Intergeneric cytoplasmic hybridization in *Cruciferae* by protoplast fusion. Mol Gen Genet 191: 244–250

Petit A, Tempe J (1978) Isolation of *Agrobacterium* Ti-plasmid regulatory mutants. Mol Gen Genet 166:147–155

Stringam GR (1979) Regeneration in leaf callus cultures of haploid rapeseed (*Brassica napus* L). Z Pflanzenphysiol 92: 459–462

Tanaka N, Hayakawa M, Mano Y, Ohkawa H, Matsui C (1985) Infection of turnip and radish storage roots with *Agrobacterium rhizogenes*. Plant Cell Rep 4:74–77

Yarrow SA, Wu SC, Barsby TL, Kemble RJ, Shepard JF (1986) The introduction of CMS mitochondria to triazine tolerant *Brassica napus* L., var. Regent, by micromanipulation of individual heterokaryons. Plant Cell Rep 5:415–418

Zambryski P, Joos H, Genetello C, Leemans J, Van Montagu M, Schell J (1983) Ti-plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J 2:2143–2150