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High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors

Maurice M. Moloney, Janis M. Walker, and Kiran K. Sharma

Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada

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ABSTRACT

An efficient procedure for obtaining transgenic *Brassica napus* plants using *Agrobacterium* binary vectors is described. The target tissue for the transformation is the cut end of cotyledonary petioles. These tissues, when cultured with their lamina intact, show a regeneration frequency of more than 80%. The cells of this cut surface, which undergo organogenesis, are very susceptible to topical infection by *Agrobacterium* . The co-cultivation method used does not require feeder layers or use of exogenously applied promoters of virulence. After 72h of infection with *Agrobacterium* the explants were transferred to selective regeneration medium. Using kanamycin (15μg cm^{-3}) for selection, transgenic plantlets emerged within 3 weeks. These plantlets which appeared on over half the explants were excised and rooted for a further 7-10 days. When the plants were large enough, leaves were taken for assay of NPT II activity using dot blots. Most of the plants surviving the selection showed substantial NPT II activity. The frequency of transformation and yield of transgenic plants was higher than in previously reported methods with this species. Southern blotting revealed that integration of the T-DNA frequently occurred in multiple copies and at multiple loci in the genome. The transgenic *B.napus* plants all grew normally and developed fertile flowers. The transgenic plants were self-pollinated and their progeny studied by two methods. The first was a single-embryo NPT II assay performed on developing seeds of these selfed-plants. The second was a leaf bleaching assay performed by selection of germinating seedlings of the selfed progeny. Both assays yielded segregation ratios consistent with the number of integration events indicated by Southern blots. The method should have broad application in studies of gene expression in the *Brassicaceae* and will be a cost-effective alternative to those seeking to improve *Brassica* crops by introduction of foreign genes.

Abbreviations NPT II - neomycin phosphotransferase; MS - Murashige-Skoog; CTAB - cetyl trimethyl ammonium bromide; CaMV - cauliflower mosaic virus; NOS - nopaline synthetase.

INTRODUCTION

Brassica napus is a particularly interesting plant species as it is both a major crop and a highly manipulable laboratory organism. Its current value worldwide as an oilseed is in excess of $5 billion per annum (F.A.O., 1988). The species is amenable to a large number of tissue culture techniques. These include whole plant regeneration from a variety of tissues such as stem explants (Stringham, 1977), leaf and root protoplasts (Newell *et al.*, 1984; Xu *et al.* 1982) and microspores (Keller and Armstrong, 1978; Chuong *et al.*, 1988). Protoplast fusion has been used to produce cybrids (Pelletier *et al.*, 1983) and intergeneric fusions such as *'Arabidobrassica'* (Gleba and Hoffman, 1980).

Offprint requests to: M. Moloney

Brassica napus has also proven to be susceptible to a variety of methods leading to the production of transgenic plants. These include the use of *Agrobacterium* vectors (Radke *et al.*, 1988; Pua *et al.*, 1987), microinjection (Neuhaus *et al.*, 1987) and electroporation (Guerche *et al.*, 1987).

While all these methods have been reported to yield transgenic *B. napus* plants, they are in general labour-intensive and inefficient. As a result, the use of *B. napus* as a model organism for gene expression studies has not been widely exploited. A few authors have used *B. napus* as a model for gene expression in seed development (Crouch *et al.*,1985 ; Radke *et al.*, 1988) or seed germination (Harada *et al.*, 1988). Nevertheless, a more efficient, routine procedure for transformation of *Brassica* species would facilitate the development of the genus as a model in plant gene expression.

In studies performed in this laboratory on *Agrobacterium*-mediated transformation, we have focused on two problems limiting the efficiency of transformation. These are: (1) access of the bacteria to wounded surfaces and (2) regenerability of different wound surfaces. We report here a transformation procedure utilizing cotyledonary explants. These cut ends of cotyledonary petioles are both highly susceptible to *Agrobacterium* -mediated gene transfer and also display very high regeneration rates, often with numerous shoots per explant. This has resulted in a convenient and efficient method for routine transformation of *B. napus*.

MATERIALS AND METHODS

Plant material

Seeds of *B. napus* cv. Westar were surface sterilized in 1% sodium hypochlorite for 20 min. The seeds were washed in sterile distilled water 3 times and planted at a density of 10 seeds per plate on Murashige-Skoog (MS) minimal organics medium (Gibco) with 3% sucrose and 0.7% phytagar (Gibco) pH 5.8. Seeds were germinated at 24°C in a 16 h light/8 h dark photoperiod at a light intensity of 60-80 $\mu Em^{-2}s^{-1}$. After 5 days the cotyledons were excised in such a way that they included ~ 2mm petiole at the base. Care was taken to eliminate the apical meristem which sometimes adheres to the petioles. The excised cotyledons were placed on MS medium, 3% sucrose and 0.7% phytagar enriched with 20 μM benzyladenine. The petioles were embedded in this solid medium to a depth of approximately 2 mm (Sharma, 1987). The cotyledons were plated at a density of 10 cotyledons per plate.

Transformation procedure

Single colonies of *Agrobacterium tumefaciens* strain EHA 101 (Hood *et al* , 1986) containing a binary plasmid were grown overnight at 28°C in AB medium (Watson *et al.*, 1975). For most of the work reported here, a binary plasmid pCGN 783 was used. This contains a CaMV 35S promoter, a neomycin phosphotransferase II (NPT II) coding region and a terminator

obtained from transcript 7 of the T_L-DNA of the *A. tumefaciens* octopine plasmid pTiAch5 (Willmitzer *et al* ,1983). The plasmid also specifies bacterial gentamycin resistance. The host strain of *Agrobacterium* , EHA 101 (Hood *et al* , 1986), is kanamycin resistant. Thus, vector EHA101 x 783 was maintained on 50µg cm^{-3} kanamycin and 50µg cm^{-3} gentamycin. A 50 µl sample of this suspension was grown overnight at 28°C in 5ml of MG/L broth (Garfinkel and Nester, 1980) supplemented with appropriate antibiotics. This bacterial suspension was pelleted by centrifugation for 15 min at 10,000 x g then resuspended in 10 ml of MS medium containing 3% sucrose and at pH 5.8. A thin film of this suspension was used to cover the base of a 5 cm petri-dish. Individual excised cotyledons were taken from the plates described above and the cut surface of their petioles was immersed into this bacterial suspension for a few seconds. They were immediately returned to the same MS plates from which they had been taken. The cotyledons were co-cultivated with the *Agrobacterium* for 72 h. No feeder layers were employed. Chemical enhancers of virulence such as acetosyringone (Stachel *et al,* 1985) were investigated, but found to give no advantage over the standard procedure. They were omitted from all transformations reported in this paper

After co-cultivation, the cotyledons were transferred to regeneration medium comprising MS medium supplemented with 20 µM benzyladenine, 3% sucrose, 0.7% phytagar, pH 5.8 and 500 mg/l carbenicillin (Pyopen, Ayerst) and 15 mg/l kanamycin sulphate (Boehringer-Mannheim). Again the petioles were carefully embedded in the agar to a depth of 2 mm. Plating density was maintained at 10 explants per plate. Higher densities reduce regeneration frequency.

Selection and plant regeneration

The explants were maintained on regeneration medium, under light and temperature conditions specified above, for 2-3 weeks. During this time many shoots appeared on over half the explants with relatively little callus formation. Some of these shoots undergo bleaching by the fourth week of culture. The remaining green shoots were subcultured onto shoot elongation medium which was the same as regeneration medium minus the benzyladenine. One or two weeks on this medium permitted the establishment of apical dominance from the shoot-clusters formed. The shoots so derived were transferred to 'rooting' medium containing MS medium, 3% sucrose, 2 mg/l indole butyric acid, 0.7% phytagar and 500 mg/l carbenicillin. No kanamycin was used at this stage as it was found that more rapid root establishment occurred without the selection agent while very few "escapes" actually succeeded in rooting after the two rounds of selection on regeneration and shoot elongation medium.

Cultivation of transgenic plants

As soon as a small root mass was obtained, the plantlets were transferred to potting mix (Redi-Earth) supplemented with osmocote fertilizer granules. The plants were grown in a misting chamber (average relative humidity 75%) for 2-3 weeks at 24°C, 16h light /8h dark photoperiod, intensity 60-80 $\mu Em^{-2}s^{-1}$. Under these conditions plants established rapidly and leaf samples were taken for NPT II assay. After 3 weeks the plants were transferred to the greenhouse and allowed to flower and set seed.

Integration and expression analyses

DNA integration

Plant DNA was prepared by the CTAB method of Rogers and Bendich (1988). Genomic blots were obtained using standard protocols (Maniatis *et al.*, 1982) onto nylon membranes (Hybond N, Amersham). These were manipulated according to manufacturer's recommendation. A 1 kb fragment comprising the coding sequence of NPT II was used as a probe and for plasmid standards (reconstructions) on the gel. Probes were labelled to $>10^9$ dpm/µg DNA using the random-primed labelling techniques of Feinberg and Vogelstein (1984).

NPT II expression

A dot-blot assay described previously by Radke *et al* . (1988) was used to determine level of NPT II activity in leaves of transformed plants.

Inheritance of NPT II activity in F1 progeny

Individual immature seeds of known transformed plants were obtained from developing siliques. These were subjected to the NPT II assay described above. Segregation patterns of NPT II activity from these seeds were thus obtained.

Seeds from selfed transformed plants were germinated on MS salts without sucrose in presence of 30µg ml^{-1} kanamycin sulphate. After seven days, plants were scored for bleaching of their first true leaves. This bleaching is characteristic of kanamycin sensitivity even though seeds of untransformed plants can germinate on this concentration of kanamycin.

RESULTS

Regeneration capacity of target tissue

The target cells for these experiments were those at the cut surfaces of cotyledonary petioles. In our laboratory, these have proven to be a vigorous source of new shoot material. In Figure 1, the development of shoots from these explants is shown. This shoot development is very rapid (2-3 weeks). The origin of these shoots has been shown by Sharma (1987) to be cells around the cut end of the petioles. This cut surface is an ideal target for *Agrobacterium* -mediated transformation as the cells undergoing organogenesis are those most readily accessed by the *Agrobacterium* treatment. It has also been demonstrated by Sharma (1987) that the explant must include most or all of the cotyledon and that the petiole must be embedded within the medium for optimal results. As is shown in Table 1, shoot regeneration frequencies for these explants without selection, are for this variety (Westar), approximately 80%. Regeneration frequencies with other varieties and many other members of the genus were similar or greater. On selection using 15µg cm^{-3} kanamycin sulphate, no shoots were obtained with explants which were not treated with *Agrobacterium* . Among treated petioles, typical regeneration frequencies of > 60% were found for all varieties tested, when selected on kanamycin. Occasionally 'escapes' were noted in transformation treatments (but not in untreated, kanamycin-selected controls). These 'escapes' appear as pale green leaflets with severe anthocyanin coloration on their lower surface. These leaflets are generally completely bleached at this concentration of kanamycin before they emerge from the agar in which the petiole has been embedded. Transformed shoots, several of which may arise on individual explants have a more normal dark green coloration and quite vigorous growth. A important aspect of this procedure is that multiple shoots often form from a single explant. However, in Table 1, we have scored an explant as having a score of '1' whether a single or multiple shoots were produced. These multiple shoots from individual explants are often due to independent transformation events at the surface.

A comparison of different procedures for transformation of *Brassica napus* indicates wide variations in transformation efficiency as measured by yield of transformed shoots obtained. Although each of the methods cited yields a large number of transformed calli, the yield of confirmed transformed plants varies between ~2% for epidermal explants (Charest *et al*, 1988) or hypocotyl explants (Radke *et al* , 1988) and 10% for stem explants (Pua *et al,* 1987). The method reported here has been optimised for factors other than target tissue eg. strain of *Agrobacterium* and concentration of selection agent. It is clear that the yield of transformed plants obtained by this procedure is significantly higher than procedures reported previously.

Expression of neomycin phosphotransferase II

All suspected transformants were assayed by a previously described dot-blot procedure (Radke *et al,* 1988). It was found that a sample of < 100mg of tissue (normally leaf) was adequate to detect substantial activity of NPT II. Figure 2 depicts a typical screen of plants assayed by this procedure after transformation with pCGN 783. In the dot-blot shown here, all the strongly expressing plants (T1, T2, T3, T5 and T7) were transformed with pCGN 783. Weaker NPT II expression was detected (T6 and T8) when vectors based on Bin 19 (Bevan, 1984) were used. This may reflect relative differences in strength between the NOS and CaMV 35S promoters as previously reported (Kay *et al*,

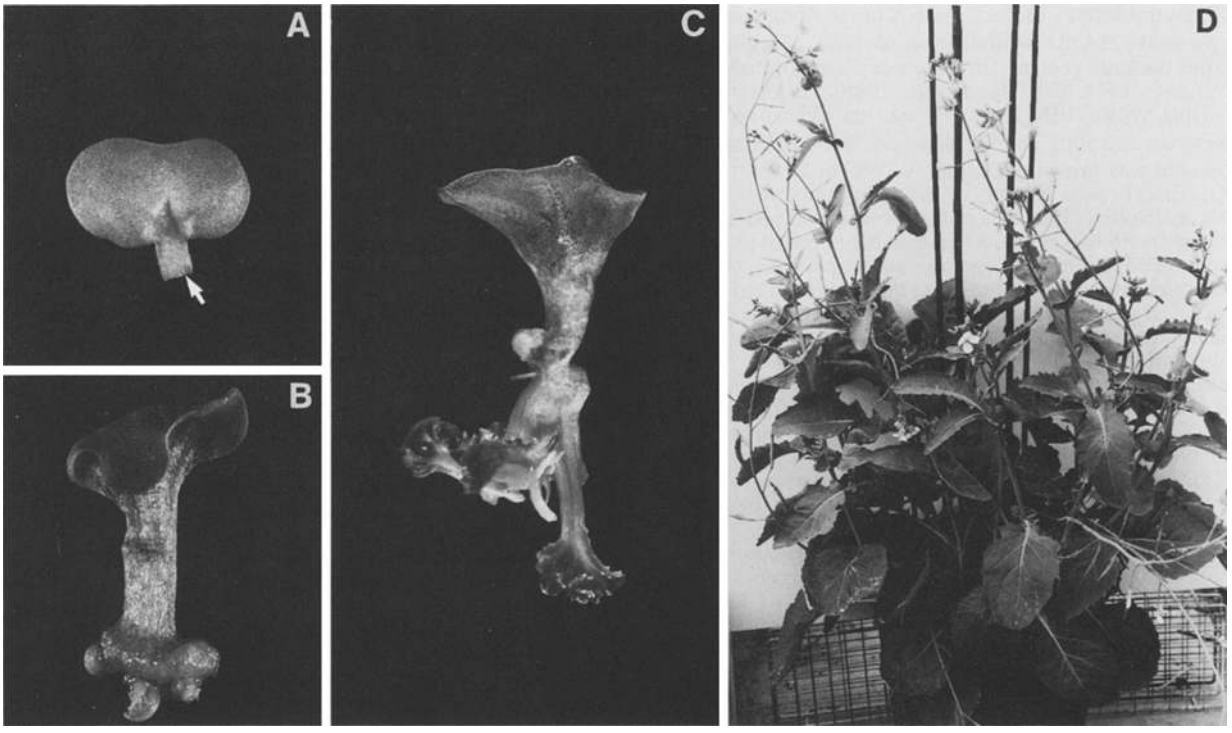

Figure 1: Development of shoots in explants of *B. napus* cotyledonary petioles. **A.** Cotyledonary explant (arrow indicates cut surface of petiole); **B.** Organogenesis in cultured cotyledonary petioles after 10-14 days; **C.** Shoot regeneration from petiole cut end; **D.** Transformant #T5 at flowering.

1987). Even with these weaker promoters, relatively few escapes' have been detected although it may be necessary to adjust the time or level of selection of these NOS-NPT II plants to obtain maximum yields.

Southern blot analysis of transformants

The Southern blot shown in Figure 3 includes all of the pCGN 783 transformants shown in Figure 2. This genomic blot of transformed and untransformed plants provides evidence of insertion of the T-DNA in each putative transformant. It also provides evidence of a range of copy number insertions into the genomes of these plants. Insertions appear to be between one and five copies. Separate digestions of T1, T2 and T3 were attempted using Hind III (data not shown), which cuts only once between the T-DNA borders of pCGN 783. These digestions indicated 4, 2 and 1 independent insertions respectively in these transformants. Besides the strong 1.6 Kb bands characteristic of an Eco RI-Bam HI excision from the genome, some scrambling of the insert also appears to have occurred. This is evident by the additional hybridizing bands in transformants T2, T5 and T9. Complex insertion patterns such as found here were also reported previously in *Brassica napus* transformants (Radke *et al*, 1988). Insert copy number frequently correlated with level of expression. This can be seen in T1, T2 and T5 all of which have multi-copy insertions and show high levels of expression in leaves (see Fig. 2). T7, which shows a much lower level of NPT II activity, was shown to have only a single copy insertion. This correlation was not absolute, however. T3 which expresses NPT II at a level similar to T2 (see Fig 2) was found to have a single copy insert (see Fig 3).

Single seed assay and inheritance studies

In order to investigate the heritability of the introduced genes, it was reasoned that a rapid screen could be performed on developing embryos of selfed, transformed plants. This would indicate those expressing NPT II and give an approximate segregation ratio. Consequently, developing seeds from T5 (see Figs 2 and 3) were excised from siliques 30 days post anthesis. At this time the seed is still green and weighs approximately 10 mg. Embryos were released from their integuments by slight

Table 1. Comparative frequencies of shoot regeneration and transformed plants reported to date using different tissue targets for the transformation of *B. napus* cv Westar.

Target tissue[1]	Regeneration frequency w/o selection (%)	Explants yielding transformed plants (%)	Reference
Cotyledonary petiole	33/40 (82.5%)	22/40 (55%)[2]	this work
Floral stem 'thin cell layer'	83/100 (83%)	12/600 (2.0%)	Charest *et al* (1988)
Hypocotyl	28/124 (22.6%)	3/122 (2.5%)	Radke *et al* (1988)
Longitudinal 'stem' section	95/100 (95%)	25/250 (10%)	Pua *et al* (1987)

1 All experiments refer to *Agrobacterium* -mediated transformation, but different vectors may have been employed.

2 Each explant was given a score of 1 if one or more shoots were produced within three weeks of culture.

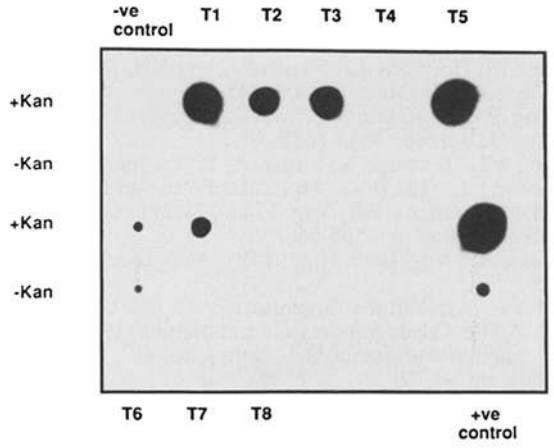

Figure 2: NPT II dot blot assay of extracts of leaves from putative transformed *B. napus* plants. Rows marked +Kan represent the result of the kanamycin labelling reaction. Rows marked -Kan represent the result of the reaction between ATP and plant extract without the substrate kanamycin. The negative control was *B.napus* cv. Westar leaves (untransformed). The positive control was a *B.napus* tumour line harbouring both T-DNA oncogenes and NPTII. T1, T2, T3, T5, T7 were transformed with pCGN783; T4, T6, T8 were treated with pBI121, a Bin 19 derived vector (Jefferson *et al.*, 1987).

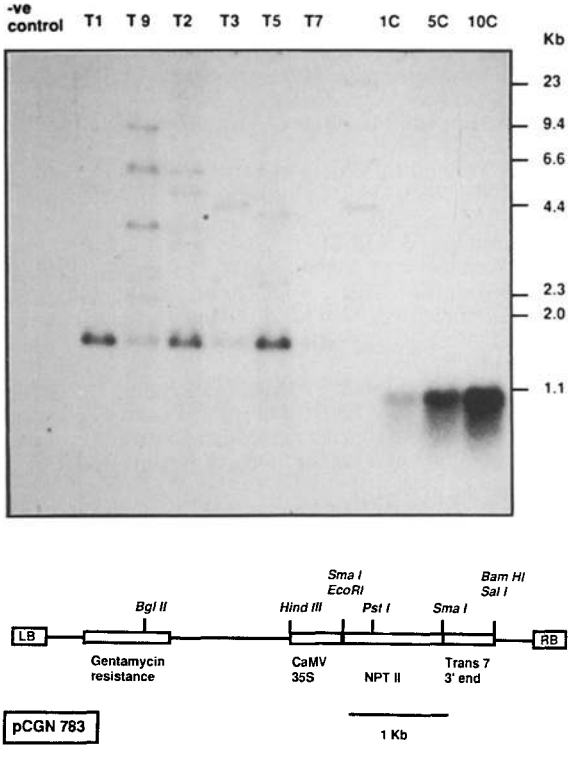

Figure 3: Southern blot analysis of EcoRI-BamHI digests of genomic DNA isolated from transformed *B. napus* plants. All transformants were obtained by integration of pCGN783. A map of pCGN 783 is given below the autoradiogram. Blots were probed with a 1 Kb fragment of NPT II coding sequence equivalent to the SmaI-SmaI region of pCGN783. 1C, 5C, 10C are reconstructions corresponding to 1, 5 and 10 copies per genome equivalent. Lanes were loaded with 10µg DNA.

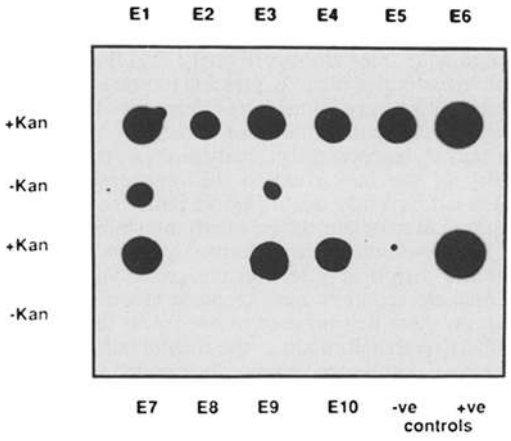

Figure 4: Single embryo assay of NPT II activity in embryos obtained from a single silique of transformant T5. Positive and negative controls were as shown for Figure 2. E8 is an apparent null; all other embryos showed high levels of activity.

pressure using forceps. The resultant individual embryos were extracted in 50 µl of extraction buffer (Radke *et al* , 1988). The extract was then centrifuged at 14000 x g for 10 min and samples prepared as for the leaf NPT II assay. A dot blot of 10 such embryos is shown in Figure 4. As can be seen, NPT II activity was very evident in these extracts.

A noteworthy feature of this assay is the variation in activity found in a family of embryos isolated from a single silique. Embryo #8 (E8) appears to be a null, while the others are all positives showing different levels of activity. This pattern of segregation suggests that the selfed parent had either 2 or 3 independent insertions. Figure 3 is consistent with this prediction in that it suggests that >1 and <5 copies of NPT II are present in the genome.

The activity detected by this method is well above the limits of detection of the assay. Consequently, it should be possible to use this as a non-destructive test for expression by taking half seeds or single cotyledons for assay. This would permit the germination of the remaining embryo to a whole plant.

In addition to these embryo assays, progeny were analysed for kanamycin resistance phenotype. This was done by germinating seeds of the T1 generation on MS salts in presence of 30µg ml^{-1} of kanamycin sulphate. It was found important to exclude sugars from this germination medium to produce consistent differences between susceptible and resistant phenotypes. Seedlings were scored for resitance to kanamycin by observing the coloration of the first true leaf. This undergoes bleaching *via* a phase of anthocyanin pigmentation over 7-10 days. All control plants showed this bleaching by 10 days. The results of this bleaching assay are given in Table 2 for transformants T2, T3, T5 and T9. Clearly, all transformants show Mendelian segregation of the introduced genes. The numbers of independent loci predicted by these assays are consistent with results obtained through Southern blotting.

Table 2: Segregation of the kanamycin resistance phenotype in first selfed generation (T1) of transformed *B.napus* plants.

Designation of parent plant	Sample size	Kan^r seedlings
T2	18	16
T3	36	29
T5	60	55
T9	46	37

DISCUSSION

The value of *Agrobacterium* -mediated plant transformation is measured primarily by the number of independent transformed plants expressing the gene of interest per explant used. This can be a function of genotype of the species to be transformed, the strain (virulence) of the *Agrobacterium* , the selectable marker, regeneration capacity of the target cells and the accessibility of the bacterium to the regenerable cells. An additional less-frequently quantified variable is the amount of labour required to maintain cultures until transformed shoots are obtained. As the selections are performed on unstable antibiotics, the longer the period of selection the greater the number of transfers that are required and the more labour-intensive the procedure. As there was no need to use feeder layers to obtain large numbers of transformants, the manipulations used were simple compared with several previously reported systems.

A key feature of this procedure is the regenerability of the cells wounded during excision of the cotyledon. These cells are mainly surface cells, readily accessible to the bacterium. Targets such as the thin cell layers from floral stems (Charest *et al,* 1988) are also highly regenerable, but as was demonstrated by Klimazewska and Keller (1985), the meristemoids that develop arise several cell layers below the epidermal surface. This greatly reduces the accessibility of the *Agrobacterium* to these regenerable sites. When longitudinal strips are taken from these tissues, a number of these regenerable regions are probably revealed. However, it is difficult to guarantee that a substantial number of these sites will be available in such epidermal strips. Thus epidermal strips may permit a high frequency of regeneration, but relatively low frequency of transformed shoots.

The analysis of integration of T-DNA by the method reported here demonstrates frequent multi-copy insertions. This is in agreement with the results of Fry *et al* (1987), who produced several *Brassica* plants with multiple inserts, in contrast to more frequent single copy insertions in the *Solanaceae.* Schmidt and Willmitzer (1989) found similar multiple inserts with *Arabidopsis* transformed populations. While this may be a feature intrinsic to the *Brassicaceae,* as suggested by Fry *et al* (1987), it may be dependent on the selection schemes and the levels or types of antibiotics used.

Our procedure appears to function with other selectable markers eg hygromycin resistance. We have also investigated several genotypes of *B. napus* including cv Global, cv Triton and a rapid cycling line, CrGC-5 (Williams and Hill, 1986), all of which are susceptible to this procedure. Our studies on other members of the *Brassica* genus using this method have shown it is effective with several members of the *Brassica* evolutionary triangle (U , 1935). Results dealing with these other species will be reported elsewhere.

The method reported here has been used to optimise frequency of transformation in a system that provides large numbers of shoots over relatively short periods. This gives a transformation scheme which allows cost-effective, routine use of *Brassica* transformation as part of basic studies in gene expression. It is hoped that use of this system will assist other workers using *Brassica* sp. to study self-incompatibility and seed-specific gene expression. It should also help breeders requiring a large population of transformants from which to commence selection of agronomically improved lines.

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