GENETIC TRANSFORMATION OF BRASSICA NAPUS AND BRASSICA RAPA

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INTRODUCTION

The genus Brassica includes one of the world's most important oilseed crops, rapeseed. Considerable effort has been made to improve its agronomic qualities by selective breeding techniques. The goals for improving this crop include increased yield and disease resistance, in addition to altering the oil content and composition.

Brassica napus and Brassica rapa constitute the majority of rapeseed production in North America. B. napus is fairly amenable to tissue culture, thus offering a system for introduction of foreign genes. Transgenic plants of B. napus obtained by Agrobacterium-mediated transformation have been reported by Pua et al. (1987), Fry et al. (1987), Radke et al. (1988) and Moloney et al. (1989). Microinjection (Neuhaus et al. 1987) and electroporation (Guerche et al. 1987) techniques have also been used to transform B. napus. However, no reports to date exist on transformation of B. rapa.

In this paper we describe an efficient and reliable system for rapeseed transformation that significantly reduces the survival of any 'escapes' (non-transgenic plants) by judiciously selecting the proper cultural conditions. Using this system, we have obtained transgenic plants in different cultivars of B. napus and have successfully transformed one cultivar of B. rapa.

MATERIALS AND METHODS

Plant Materials

The cultivars used in this study included B. napus cv. Westar and Profit and B. rapa cv. Parkland. Seeds were surface sterilized with 1.05% sodium hypochlorite for 20 minutes and rinsed 3 times with sterile distilled water. These seeds were aseptically germinated on basal medium (BM) in 20x100mm petri dishes for 4-6 days. The BM consisted of Murashige and Skoog (1962) macro- and micro-elements, iron as 40 mg/l FeNa2EDTA, and the following constituents (mg/l): myo-inositol 100; nicotinic acid 0.1; pyridoxine HCl 0.1; thiamine HCl 0.02; glycine 0.4; sucrose 30,000; Difco bacto agar 8,000. The seedlings were grown at 25°C with a photoperiod of 16 hours. Hypocotyl segments (2-3 mm) were excised from 4-6 days old seedlings and pre-treated for 24 hours on BM or Gamborg's B5 (Gamborg et al. 1968) medium containing α-naphthaleneacetic acid (NAA) at 5 mg/l or 2,4-dichlorophenoxy acetic acid (2,4-D) at 1 mg/l (callusing medium) depending on the cultivar used. A sterile filter paper was placed on the medium prior to treatment.

Cotyledon explants were excised from 6 day old seedlings ensuring that 2-3 mm of the petioles were left intact with the lamina. These explants were pre-treated for 1 day on BM containing 6-benzylaminopurine (BAP) at 4 mg/l. The cotyledonary petioles were then embedded in the medium.

Vectors

The vectors used in these experiments were pH602 or pH610 in the Agrobacterium tumefaciens strain Z707S, which is a

disarmed C58 derivative. Both vectors contain the hygromycin phosphotransferase (HPT) gene that confers resistance to the antibiotic hygromycin. The plasmid pH602 contains the HPT gene driven by the CaMV 35S promoter. The second plasmid pH610, which is a derivative of pH602, also contains a truncated Bacillus thuringiensis toxin gene driven by the mannopine promoter (Adang et al. 1987).

Transformation Procedure

The hypocotyl segments and the cotyledon explants were treated with the Agrobacterium solution (diluted to 10^8/ml with liquid basal medium) for 30 min. and then placed onto the callusing medium for 2-3 days of co-cultivation. The hypocotyl tissues were transferred to the callusing medium containing 500 mg/l carbenicillin and 5-10 mg/l hygromycin. The cultures were maintained at 22±2°C with 16 hrs photoperiod. After 7 days, the hypocotyl segments were transferred to shoot regeneration medium BM or B5 containing 1-4 mg/l BAP, 0-4 mg/l zeatin, 2.5-10 mg/l silver nitrate (AgNO3), 500 mg/l carbenicillin and 5-10 mg/l hygromycin; the concentrations were dependent on the cultivar and the vector being used. The callusing and regeneration media were solidified with 0.5% Agarose (SeaKem) or 0.2% Gelrite. The tissues were transferred to fresh selection medium every 3 weeks. Callusing occurred after 1-3 weeks and shoots were formed after 3-6 weeks. These shoots were then transferred to BM containing 0.01-0.1 mg/l BAP and carbenicillin for elongation and later were rooted on BM with 0.1 mg/l indole butyric acid (IBA).

After treatment with Agrobacterium, cotyledon explants were transferred to selection medium containing BAP (4 mg/l), carbenicillin and hygromycin at the same levels used for hypocotyls. Shoots formed on the cotyledonary petiole were visible within 3-6 weeks and were cultured using the same protocol as for hypocotyls.

Molecular and Biological Assays

All plants were assayed and determined to be transgenic by the following molecular and biological assays:

Polymerase Chain Reaction - A linked reverse transcription and PCR assay modified from Frohman et al. (1988) was used to analyze small amounts of leaf tissue for the presence of RNA transcripts from the HPT gene.

Hygromycin Phosphotransferase (HPT) Activity - A rapid dot-blot assay based on the methods of Reiss et al. (1984) developed for antibiotic phosphotransferases was used to assay HPT activity.

Leaf Disc Assay - Small leaf sections (2-3 mm square) isolated from shoots obtained on selection medium were cultured on BM containing 4 mg/l BAP, 0.5 mg/l NAA and 10 mg/l hygromycin for 4 weeks.

Southern Analysis - The presence of the HPT gene in plant DNA was determined by Southern analysis (Southern,1975) using an internal DNA segment from the structural gene as a probe.

Genetic Analysis

Transgenic (Ro) plants were self-pollinated and the R1 seeds were surface sterilized and germinated on BM containing 20 mg/l hygromycin for identifying those seedlings that expressed the HPT gene. Resistance or sensitivity of the seedlings was scored after 3 weeks.

RESULTS AND DISCUSSION

Transformation

The response of several different tissue types including hypocotyl segments, longitudinal stem segments and cotyledonary petioles to Agrobacterium-mediated transformation was compared. All explant sources yielded transgenic plants. However, they varied in efficiency, reliability and transformation frequency. Transgenic plants were regenerated from longitudinal stem segments of plants grown in culture. Although this transformation system is efficient, the supply of sufficient explant material is a limiting factor and the continuous maintenance of healthy donor shoots in culture is time consuming. Stem explants from plants grown in the greenhouse or in an environmental chamber showed a wide variability in the regeneration frequency, which may be attributed to the physiological state of the donor plants.

Transgenic shoots were obtained from cotyledonary petioles at a frequency of 0-10%, with a wide variability between experiments. These differences may be dependent on the regeneration potential of the cells at the cut cotyledonary petiole surface, i.e. only certain cells may be competent for regeneration and transformation.

We have selected hypocotyl segments as the preferred donor tissue because we can consistently regenerate transgenic plants. It further offers the advantage that large amounts of donor material are available within 4-5 days of seed germination. After co-cultivation of the hypocotyls with Agrobacterium, the resultant calli were transformed at a high frequency (70-80%). These calli gave rise to transgenic shoots at a frequency of up to 13%. Of the shoots regenerated from calli on the selection medium, more than 90% were transgenic. One week treatment on callusing selection medium (2,4-D or NAA) and then transfer to the regeneration medium was essential for obtaining a high transformation frequency. Formation of transgenic shoots was significantly reduced by culture of hypocotyl tissues on a hormone combination that promoted both callusing and regeneration.

A higher frequency (40-60%) of shoot regeneration was obtained by incorporating AgNO3 (2.5-10 mg/l) in the culture medium as compared to the frequency (10-15%) obtained on medium lacking AgNO3. Timing of AgNO3 incorporation was equally important, being more effective when incorporated only in the regeneration medium and not in the callusing medium (Table 1).

Table 1. Influence of silver nitrate addition on shoot regeneration from hypocotyl sections of B. napus cv. Westar.

Expt.	AgNO3 in callusing medium	AgNO3 in regeneration medium	Shoot regeneration frequency
H6	+	+	30
H6	-	+	56
H8	+	+	2
H8	-	+	42

The presence of agarose or gelrite in the media affected both the transformation frequency and the rate of regeneration. Table 2 shows that 3-7 times more shoots regenerated using gelrite or agarose in the medium as compared to agar. Shoots obtained from hypocotyl callus after 4 weeks on selection medium are shown in

Fig. 2. The transgenic shoots appeared earlier on the agarose containing medium. Rooted plantlets were formed within 3 months of explant co-cultivation as compared with 4-5 months for those cultured on agar-containing medium.

Table 2. Effect of gelling agent on transformation frequency from hypocotyl sections of B. napus cv. Westar.

Gelling agent	# Segments treated	# Segments with shoots	Transformation frequency
Agarose	400	52	13
Gelrite	400	20	7
Agar	400	7	1.7

The use of hygromycin provided a very 'tight' selection system and significantly reduced the possibility of any 'escapes'. Over 90% of the plants that came through the selection procedure were transgenic. Direct selection, i.e. culturing hypocotyl segments on hygromycin medium directly after co-cultivation was essential for obtaining primarily transgenic plants. Delayed selection by culturing the tissues on non-selective medium for 3-7 days before transfer to selection medium allowed more 'escapes' to form. We have regenerated more than 200 transgenic plants using this procedure and these plants show normal morphological characteristics.

Genotype effect

B. napus cultivars Westar and Profit gave a higher transformation frequency than B. rapa cultivar Parkland. Transformation frequencies up to 13% (average 5%) were obtained with Westar and Profit, whereas a maximal transformation frequency of 2% was possible with Parkland. Hypocotyl callus from Westar and Profit continued to regenerate transgenic shoots on selection medium even after the first 6 weeks. No additional shoots were regenerated from Parkland callus after 3 weeks on selection medium. There was no difference in transformation frequency using the different vectors (Table 3). This procedure has been used to successfully transform 3 different cultivars of 2 species of Brassica. This indicates that other Brassica species may be amenable to transformation using this same procedure or a slight modification of the same.

Table 3. Influence of genotype on transformation frequency from hypocotyl tissues.

Expt.	Vector	Selection (Hyg. mg/l)	#Segments treated	#Segments with shoots	Transformation frequency
Westar					
1.	pH602	5-10	198	12	6.0
2.	pH602	5-10	200	11	5.5
3.	pH610	10	105	7	6.6
Profit					
1.	pH602	5-10	500	27	5.4
2.	pH602	5-10	323	6	1.9
3.	pH610	5-10	250	12	4.8
Parkland					
1.	pH602	10	350	2	0.5
2.	pH602	10	371	2	0.5
3.	pH602	10	358	5	1.4

Expression and integration

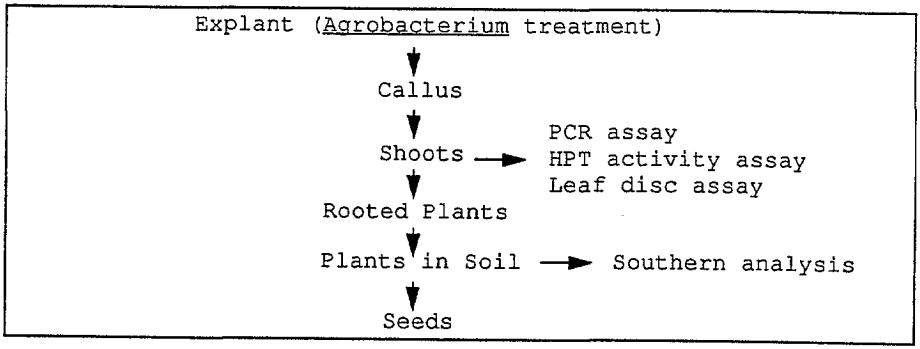
Transformation was confirmed by the molecular assays (PCR assay, Hygromycin phosphotransferase activity assay and Southern analysis) and the biological assay (Leaf disc assay). Over 90% of the shoots which regenerated under hygromycin selection were transformed as determined by these analyses performed on leaf samples (Fig. 1).

The PCR assay was performed on RNA isolated from a small amount of leaf tissue. The primer sequences yield a 600 bp DNA fragment when the RNA transcripts of the HPT gene are present.

The level of HPT activity was determined by the dot blot assay. As shown in Fig.3 there were varying levels of HPT activity in the transgenic plants.

For the leaf disc assay, callusing of the leaf tissues on hygromycin containing medium was determined after 4 weeks. Explants from transgenic shoots expanded and formed a dark green callus which regenerated roots and/or shoots (Fig.4). Tissues from non-transgenic control plants turned white or brown and showed no expansion.

Figure 1. Transformation Procedure



The above assays are very useful for early detection of transgenic plants because these require only 50-100 mg of leaf tissue. Results from leaf disc assays and Southern analyses correlated 100%; all the shoots determined positive with leaf disc assay were positive with Southern blots. Results from the expression assays (PCR and HPT activity) correlated 80-90% with Southern analysis. About 10-20% of the shoots tested negative by PCR and HPT activity assays were positive by Southern blots (Table 4).

Southern hybridizations of the DNA from thirty two plants confirmed the insertion of T-DNA in each transgenic plant; all DNA samples hybridized to the probe for the HPT gene (Fig. 5). Plants regenerated from the same callus had an identical pattern of restriction fragments, indicating they arose from the same transformation event.

Genetic analysis

Genetic inheritance of the HPT gene was determined by scoring the progeny of the transgenic plants for tolerance to hygromycin. Seeds (R1) from five independent transgenic plants were analyzed for inheritance of T-DNA by growing seeds from these plants on selection medium containing hygromycin. Inheritance data shows that the hygromycin resistance marker segregates as one or two Mendelian loci (Table 5).

Table 4. Comparison of molecular and biological assays for transgenic plants.

Plant Number	Southern Analysis	PCR Assay	HPT Activity	Leaf disc Assay
HH2-1	+	+	+	+
HH2-2	+	+	-	+
HH2-3	+	+	+	+
HH2-4	+	+	+	+
HH2-5	+	+	+	+
HH2-6	+	+	+	+
HH2-18	+	+	+	+
HH2-19	+	+	+	+
HH2-36	+	+	+	+
HH4-1	+	ND	+	+
HH4-3	+	ND	+	+
HH5-101	+	ND	+	+
HH7-13	ND	+	+	+
HH7-25	ND	-	+	+
HH7-26	+	+	+	+
HH7-27	+	-	+	+
H10-3	+	-	ND	+
H10-4	+	-	ND	+
HH14-59	+	+	+	+
HH17-48	+	-	ND	+
S5-32	+	+	+	+
HH18-53	ND	+	-	+
HH18-62	ND	+	-	+
HH18-69	ND	+	+	+

ND - Not determined

Table 5. Genetic Analysis of R1 progeny from 5 transgenic plants of B. napus cv. Westar.

Plant#	Hygromycin Resistant	Hygromycin Sensitive	Expected Ratio	X^2
HH2-1	26	3	15:1	0.85ns
HH2-2	36	23	9:7	0.52ns
HH3-1	34	26	9:7	0.003ns
HH2-4	35	15	3:1	0.66ns
H10-2	27	13	3:1	1.2ns

CONCLUSIONS

The transformation method described in this paper is highly efficient and reliable for obtaining primarily transgenic plants. Furthermore, this is the first report on the successful transformation of B. rapa. These methods may have wide applicability for transforming other species of Brassica. We are utilizing these techniques for the incorporation of genes for desired agronomic traits into rapeseed.

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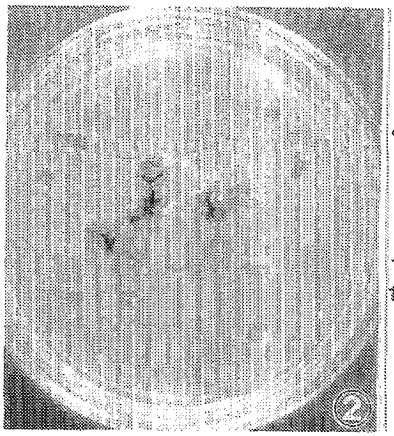

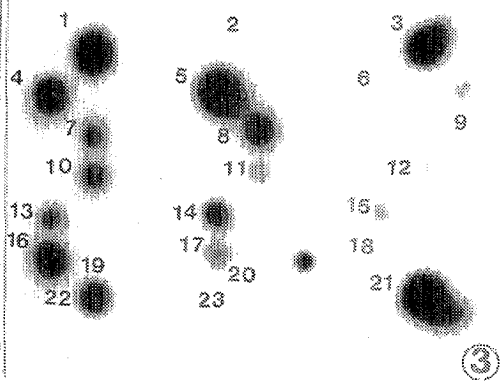

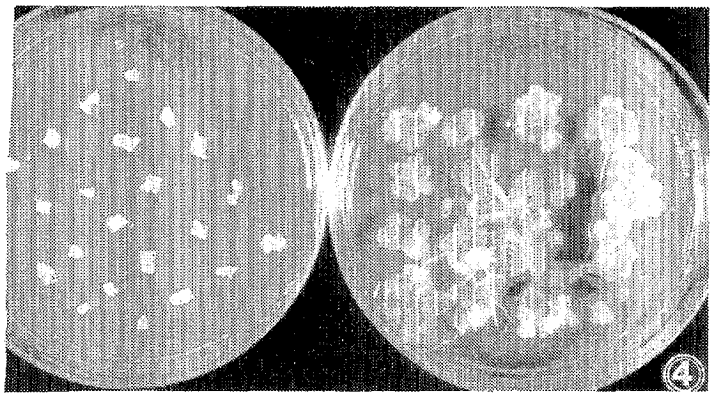

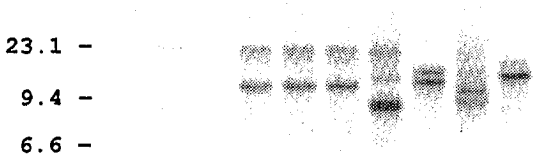

Figures 2-5. Transformation in B. napus cv. Westar. Fig. 2. Shoots regenerated on hypocotyl callus after 4 weeks on selection medium. Fig. 3. Hygromycin activity dot blot assay of leaf tissue extracts; numbers denote individual transformants. Fig. 4. Leaf tissues on medium containing 10 mg/l hygromycin. Left plate: non-transformed control. Right plate: transgenic plant. Fig. 5. Southern analysis of DNA from regenerated transformants and non-transformed control. Lane 1 and 10 molecular weight markers, 2 non-transformed control, 3,4 and 5 transformants from the same callus, 6,7,8 and 9 transformants from different callus tissues.