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Transformation of *Brassica napus* and *Brassica oleracea* Using *Agrobacterium tumefaciens* and the Expression of the *bar* and *neo* Genes in the Transgenic Plants

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ABSTRACT

An efficient and largely genotype-independent transformation method for *Brassica napus* and *Brassica oleracea* was established based on *neo* or *bar* as selectable marker genes. Hypocotyl explants of *Brassica napus* and *Brassica oleracea* cultivars were infected with *Agrobacterium* strains containing chimeric *neo* and *bar* genes. The use of $AgNO_3$ was a prerequisite for efficient shoot regeneration under selective conditions. Vitrification was avoided by decreasing the water potential of the medium, by decreasing the relative humidity in the tissue culture vessel, and by lowering the cytokinin concentration. In this way, rooted transformed shoots were obtained with a 30% efficiency in 9 to 12 weeks. Southern blottings and genetic analysis of S1-progeny showed that the transformants contained on average between one and three copies of the chimeric genes. A wide range of expression levels of the chimeric genes was observed among independent transformants. Up to 25% of the transformants showed no detectable phosphinotricin acetyltransferase or neomycin phosphotransferase II enzyme activities although Southern blottings demonstrated that these plants were indeed transformed.

The genus *Brassica* includes several very important crop species. *Brassica napus* is the most important oilseed crop in Northern Europe, Canada, and China, and *Brassica oleracea* encompasses important vegetables. In the last 30 years a great deal of effort has gone into improving the quality of *B. napus* using both classical breeding and several tissue culture techniques. Genetic engineering can potentially be used as a method to add specific characteristics to existing varieties. The latter would be most practical if efficient, genotype-independent, and reproducible transformation and regeneration techniques were available. Recently, several reports concerning the transformation of *B. napus* using *Agrobacterium tumefaciens* appeared (3, 9, 21, 23). However, in these transformation protocols only the spring variety Westar was used as starting material. In our laboratory these methods could not be extended to most other spring varieties and none of the tested winter varieties.

In this paper we describe the different parameters important for a genotype-independent transformation method for *B. napus* and *B. oleracea* using chimeric *neo* and *bar* genes as selectable marker genes. Hypocotyl explants from sterile seedlings were chosen as starting material. The chimeric *neo* and *bar* genes were used as marker genes. The *bar* gene codes for the enzyme PAT,[1] which inactivates the herbicide phosphinotricin (glufosinate) by acetylating it (18). Phosphinotricin is a glutamate analog that inhibits glutamine synthetase. The inhibition results in the accumulation of NH_4^+ which is toxic for the plant cell. Transgenic plants expressing a chimeric *bar* gene are resistant to high doses of phosphinotricin (6, 7). The expression of the chimeric genes in the transgenic *B. napus* plants was studied.

MATERIALS AND METHODS

Agrobacterium Strain

The *Agrobacterium* strain used was C58C1Rif (pMP90) (pGSFR780A) (6, 14).

Plant Materials

The *Brassica* species and varieties used are summarized in Table I. Seeds were soaked in 70% ethanol for 2 min, then surface-sterilized for 10 min in a sodium hypochlorite solution (with 30 g/L active chlorine) containing 0.1% Tween 20. Finally, the seeds were rinsed three times in sterile tap water, followed by a fourth rinse in sterile distilled water. They were germinated on A1 medium (Table II) at low light intensity (500 lux) for 3 d followed by a higher light intensity (2000 lux) for an additional 10 d. The seedlings were grown at 24°C with a daylength of 16 h. A mixture of 'Lumilux White' and 'Natura' from Osram (FRG) was used.

Media

The media are summarized in Table II.

Transformation, Selection, and Regeneration

Twelve to 14 d after sowing, the hypocotyls were cut in 7 mm segments. About 15 hypocotyl explants were floated in a 9 cm Petri dish on 10 mL of infection medium: A2 for *Brassica oleracea* and A3 for *Brassica napus*. To each Petri dish, 20 μL of a late log *Agrobacterium* culture in MinA

[1] Abbreviations: PAT, phosphinotricin acetyltransferase; NPTII, neomycin phosphotransferase II; PVPP, polyvinylpolypyrrolidone; BAP, 6-benzylaminopurine.

medium (17) was added. These plates were incubated at low light intensity (500 lux). After 2 d the hypocotyl segments were washed with A2 medium containing 500 mg/L carbenicillin, patted dry on filter paper, and placed on selective medium: A4 for *B. oleracea* and A5 for *B. napus*. As selective agents 50 mg/L kanamycin-SO_4 or 20 mg/L D+L-phosphinotricin (NH_4^+-salt) were used. The Petri dishes (2.2 cm high and 14 cm in diameter) were sealed with 'Urgo Pore Tape' (Urgo, Chenove, France) and incubated at 2000 lux (a mixture of 'Lumilux White' and 'Natura' from Osram, FRG) with a daylength of 16 h at 24°C. Three to 6 weeks after selection, calli with small shoots were formed. Entire calli with the shoots were removed from the hypocotyl explants and transferred to medium A6 (without selection). The shoots grew out and were isolated. Healthy shoots (2 cm or larger) were transferred directly to rooting medium A8. If the shoots appeared abnormal (either vitrified, 'stumpy,' or otherwise) or were still too small (less than 2 cm), they were cultured further on medium A7. Once these shoots had obtained a more or less normal phenotype they were transferred to rooting medium A8. Rooted shoots were propagated (either the top shoot or stem pieces with an axial knob) on medium A1 or transferred directly to the greenhouse.

Vernalization

The winter cultivars of *B. napus* were vernalized at 4°C for 12 weeks at low light intensity (500 lux).

Herbicide Applications

Nonflowering plants of about 20 to 30 cm high, or 6 weeks old seedlings, were placed in 1 m^2 and sprayed with a 1% aqueous solution of the commercial preparation Basta containing 20% D+L-glufosinate (phosphinotricin) (Hoechst AG, FRG) from all four sides using a Badger (Badger Air-Brush Co/IL, USA) air-brush line.

PAT Activity Assay

The PAT assays were done as described (7). Tissue (100 mg) was ground in the presence of 100 μL of extraction buffer (25 mM Tris-HCl [pH 7.5], 1 mM Na_2-EDTA, 0.15 mg/mL PMSF, 0.3 mg/mL BSA, 0.3 mg/mL DTT) to which 5 mg PVPP was added. The extract was cleared in an Eppendorf centrifuge for 5 min. To 12 μL of a 10-fold diluted extract were added 1 μL of a 3 mM phosphinotricin stock, 1 μL of a 2 mM acetylcoenzyme A (AcCoA) stock, and 2 μL of ^{14}C-labeled AcCoA (58.1 mCi/mmol; NEN). The reaction mixture was incubated at 37°C for 30 min, and 3 to 6 μL was spotted on a silicagel TLC plate. Ascending chromatography was carried out in a 3 to 2 mixture of 1-propanol and NH_4OH (25% NH_3). [^{14}C] was visualized by autoradiography (XAR-5-Kodak film overnight).

Table I. *Brassica Species and Cultivars*

Species	Variety	Cultivar	Supplied by
napus	oleifera	Westar[a]	Allelix (Canada)
		R8494[b]	Hilleshog (Sweden)
oleracea	botrytis	Walcheria	Aveva (Belgium)
		Andersen	Clause (France)

[a] Spring variety. [b] Winter variety.

Table II. *Media*

Medium[a]	Modified as Compared to Basal Medium
A1	Half-concentrated salts, no vitamins, 2% sucrose, 0.8% agar
A2	0.5 g/L Mes (pH 5.5), 0.1 mg/L NAA, 1 mg/L BAP
A3	A2 + 0.01 mg/L GA3
A4	0.5 g/L Mes (pH 5.7), 40 mg/L adenine-SO_4, 0.5 g/L PVP, 0.5% agarose, 0.1 mg/L NAA, 1 mg/L BAP, 500 mg/L carbenicillin, 2–10 mg/L $AgNO_3$[b]
A5	A4 but + 2% sucrose, 0.01 mg/L GA_3
A6	0.5 g/L Mes (pH 5.7), 40 mg/L adenine-SO_4, 0.5 g/L PVP, 2% sucrose, 0.5% agarose, 0.0025 mg/L BAP, 250 mg/L carbenicillin
A7	Half-concentrated salts, no vitamins, 1% sucrose, 0.5% agarose, 0.0025 mg/L BAP, 100 mg/L carbenicillin
A8	100 mL half-concentrated salts, no vitamins, 1% sucrose (pH 2.5), 0.1 mg/L IBA, 100 mg/L carbenicillin added to 200 mL vermiculite in 1 L vessels[c] (final pH 6.2)

[a] Basal medium in all cases was Murashige and Skoog medium (19). [b] $AgNO_3$ was added after the medium was autoclaved. [c] The medium and the vermiculite were autoclaved separately.

NPTII Activity Assay

The extraction of the leaf material was done as in the PAT assay. The NPTII assay was carried out according to McDonnell *et al.* (16). As reaction buffer, 50 mM Tris-HCl (pH 7.1), 50 mM $MgCl_2$, 200 mM NH_4Cl was used.

Ammonia Determination in Plant Extracts

Plants of 20 to 30 cm high were treated with 8 L Basta/ha. After 6 to 24 h, 250 mg leaf material was extracted in 1 mL of water containing 50 mg PVPP. Insoluble material was pelleted in an Eppendorf centrifuge for 5 min. Of the supernatant, 200 μL was diluted with 800 μL water. The ammonia was determined using the method of Weatherburn (27). To 1.5 mL of reagent A (5 g phenol, 25 mg sodium nitroprusside per 500 mL solution) 20 μL of the diluted plant extract was added, followed by 1.5 mL of reagent B (2.5 g sodium hydroxide, 1.6 mL of sodium hypochlorite with 13% available chlorine, to 500 mL of water). The reaction mixture was incubated at 37°C for 15 min. The absorbance was measured at room temperature at 625 nm. The standard curve was made using NH_4Cl in concentrations ranging from 0 to 6 μg

NH_4^+-N and 20 μL leaf extract from a nontreated control plant was added to the reaction mixtures.

Plant DNA Isolation and Southern Analysis

DNA was isolated from leaf tissue as described (8). To 15 mL of extraction buffer 50 mg PVPP was added. Five μg DNA was digested with restriction enzymes and electrophoresed in an 0.8% agarose gel, transferred to nylon Hybond-N filters, and hybridized with multiprime labeled DNA (Amersham) as described in the Amersham manual for the use of Hybond-N filters. A purified *Bam*HI fragment containing the *bar* gene from the plasmid pGSFR780A (Fig. 1A), and a *Bam*HI-*Hin*dIII fragment containing the *neo* gene from the plasmid pKM109 (24) were used as probes.

RESULTS

Tissue Culture Techniques

Hypocotyl explants of *Brassica napus* and *Brassica oleracea* were infected as described in "Materials and Methods." The *Agrobacterium* strain used for infection contained both a chimeric *neo* and *bar* gene. Selection was done on 50 mg/L kanamycin-SO_4 or 20 mg/L D+L-phosphinotricin (NH_4^+-salt). Table III summarizes the frequencies with which rooted transformed *B. napus* shoots were obtained after selection on the different media. Under all the described conditions, about 100% of the hypocotyl explants formed transformed calli after a few weeks. However, the efficiency of shoot formation and the amount of shoots that were able to root differed considerably depending on the media on which the calli were selected. From Table III it is clear that MS medium is superior to B5 medium, and that the addition of $AgNO_3$ to the medium is an absolute prerequisite to obtain transformed shoots. For the winter varieties tested, the use of $AgNO_3$ is even required in nonselective conditions (data not shown). The optimal sugar source and concentration was found to be 2% sucrose, on which many shoots were formed that could easily be rooted (see "Discussion"). Carbenicillin or triacillin at 500 mg/L were found to be optimal to eliminate the *Agrobacterium*, in contrast to cefotaxime, which was inhibitory to callus formation and shoot induction. As described previously (6), the addition of 250 mg/L carbenicillin in $AgNO_3$-containing medium is recommended, even when no *Agrobacterium* infection was done. The addition of carbenicillin prevents the medium from turning brown and eliminates the toxic effects of prolonged use of Ag^+ on the plant tissue.

The conditions developed for *B. napus* could, with a few modifications, be extended to *B. oleracea* (Table IV). GA_3 had to be omitted from the medium since it caused vitrification of the shoots, resulting in a poor root formation. $AgNO_3$ was omitted under nonselective conditions, since it inhibited shoot formation (data not shown). However, under selective conditions 2 to 5 mg/L $AgNO_3$ were necessary to obtain efficient shoot induction. Also on 3% sucrose more shoots were obtained than if 2% sucrose was used.

Efficient root formation was possible only from nonvitrified shoots. For this, conditions were set up that counteract or reverse vitrification. Small shoots or even calli with meristems, grown on medium A4 or A5, were transferred to medium A6 containing a low concentration of BAP (0.0025 mg/L). This step had two advantages. First, the shoots could develop further without becoming necrotic (antisenescence activity of BAP). Second, copies of the same shoots were obtained (see below). Once the shoots were nicely developed, they were cut from the callus and transferred to medium A7 containing only 1% sucrose. The low BAP concentration had still to be used. Little shoots often died or dedifferentiated again to callus when they were placed on medium containing no hormones or only auxins. On medium A7 the shoots proliferated and formed clusters of shoots. These complex shoots were separated to single shoots and the callus tissue was cut away. Small shoots (less than 2 cm) were put again in the medium A7, while the bigger shoots were transferred to medium A8 solidified with vermiculite and containing 0.1 mg/L IBA.

Table III. *Selective Media Tested in Transformation Experiments with B. napus*

Transformation rates were measured as the percentage of infected hypocotyl explants that gave rooted transformed shoots. The numbers are an average of the frequencies obtained in five independent transformation experiments. In each experiment 50 explants were used per condition and per selective agent (PPT or K_m).

Basic Medium	Hormones			$AgNO_3$	Carbon Source	Transformation			
						R8494		Westar	
	BAP	NAA	GA3			PPT[a]	K_m[b]	PPT	K_m
				mg/L		%			
B5[c]	1	0.1	0.01	10	2% sucrose	4	6	4	8
MS	1	0.1	0	10	2% sucrose	9	14	15	17
	1	0.1	0.01	0	2% sucrose	0	0	0	2
	1	0.1	0.01	2	2% sucrose	9	13	13	19
	1	0.1	0.01	5	2% sucrose	29	39	30	41
	1	0.1	0.01	10	2% sucrose	28	36	25	31
	1	0.1	0.01	10	3% sucrose	13	18	11	16
	1	0.1	0.01	10	1.75% glucose	16	19	14	20

[a] 20 mg/L phosphinotricin. [b] 50 mg/L kanamycin-SO_4. [c] Gamborg B5 medium (10).

Table IV. *Selective Murashige-Skoog Media Tested in Transformation Experiments with B. oleracea*

Transformation rates were measured as the percentage of infected hypocotyl explants that gave rooted transformed shoots. The numbers are an average of the frequencies obtained in five independent transformation experiments. In each experiment 25 explants were used per condition and per selective agent (PPT or K_m).

Hormones			$AgNO_3$	Carbon Source	Transformation			
					Walcheria		Andersen	
BAP	NAA	GA3			PPT[a]	K_m[b]	PPT	K_m
			mg/L	*% sucrose*	%			
1	0.1	0	0	3	8	9	10	11
1	0.1	0	2	3	28	26	25	27
1	0.1	0	5	3	24	25	30	29
1	0.1	0	10	3	10	11	13	14
1	0.1	0	2	2	21	20		
1	0.1	0	5	2	19	19		
1	0.1	0.01	2	3	18	20		
1	0.1	0.01	5	3	19	16		

[a] 20 mg/L phosphinotricin. [b] 50 mg/L kanamycin-SO_4.

The use of vermiculite instead of agar, agarose, gelrite, or combinations of these gelling agents, was a prerequisite to obtain nonvitrified plants. Shoots that were vitrified often formed new-grown parts on medium A8 that had a completely normal phenotype. These healthy parts could be cut off and put on medium A8. Nonvitrified shoots formed roots with many roothairs after a few weeks. This was not the case in agarose (0.5%) solidified medium, in which root meristems were formed, but there was no root elongation. Also in agar (1%) solidified medium, there was poor root elongation, and these roots either contained no roothairs or if so the roots laid on the medium. Probably, there is in the agar medium a lack of aeration. Once the shoots were rooted they could easily be propagated on medium A1 or transferred directly to the greenhouse. The rooted plants obtained in this way all survived transfer to the greenhouse.

Leaf Disc Assay

Leaf pieces from plants obtained after kanamycin or phosphinotricin selection were placed on medium A5 containing 20 mg/L phosphinotricin or 50 mg/L kanamycin-SO_4. After 2 weeks a range of resistance levels could be seen (Table V; Fig. 1B), varying from a good reaction (leaf pieces stayed green and formed healthy calli) to a poor reaction (the leaf pieces contained many necrotic regions and formed very few calli). The control leaves always turned completely yellow and never formed calli on the selective media. A complete correlation between the resistances to both kanamycin and phosphinotricin was found. Plants strongly resistant to one antibiotic were also strongly resistant to the other; likewise, plants weakly resistant to one antibiotic were weakly resistant to the other.

NPTII and PAT Activity

The NPTII and PAT activities of the transgenic plants were correlated with the resistance levels found in the leaf disc assay (Table V; Fig. 1, C and D). Plants with high PAT or NPTII activities were very resistant in the leaf disc assay. However, plants that had a clear although weaker resistance in the leaf disc assay had no detectable enzyme activities. Of 140 plants positive in a kanamycin selection test varying from leaf pieces with small necrotic regions, but with green and healthy calli, to completely necrotic leaf pieces with yellow calli, 37 were found to be negative in the NPTII and PAT assays. Southern hybridizations demonstrated that these plants were indeed transformed and contained both the *neo* and *bar* genes (see below). Thus, the leaf disc assay is much more sensitive and reliable than the enzymatic assays, and was relied upon in further experiments.

Resistance to the Herbicide Basta

Transgenic plants of *B. oleracea* and *B. napus* were sprayed with doses equivalent to 8 L Basta/ha. Control *Brassica* plants of the four cultivars were effectively killed with doses equivalent to 2 L Basta/ha. Plants with high PAT activities were completely resistant. However, plants with lower enzyme activities showed damage (Table V). Because the measurement of the ammonia production in the leaf tissue after Basta treatment is a very sensitive and quantitative assay (7, 28), ammonia determinations were done 6 and 24 h after treatment of the transgenic plants with doses equivalent to 8 L Basta/ha (Table V). In general, a correlation was found between the amount of ammonia accumulated and the resistance seen in the leaf disk assay (Table V).

Southern Hybridization Analysis

Southern hybridization analysis were done of transgenic plants obtained by kanamycin or phosphinotricin selection. The copy number of the chimeric gene was determined by using those restriction enzymes that cut the DNA in such a way that a border fragment composed of plant DNA and the chimeric gene was created. A ^{32}P-labeled purified DNA frag-

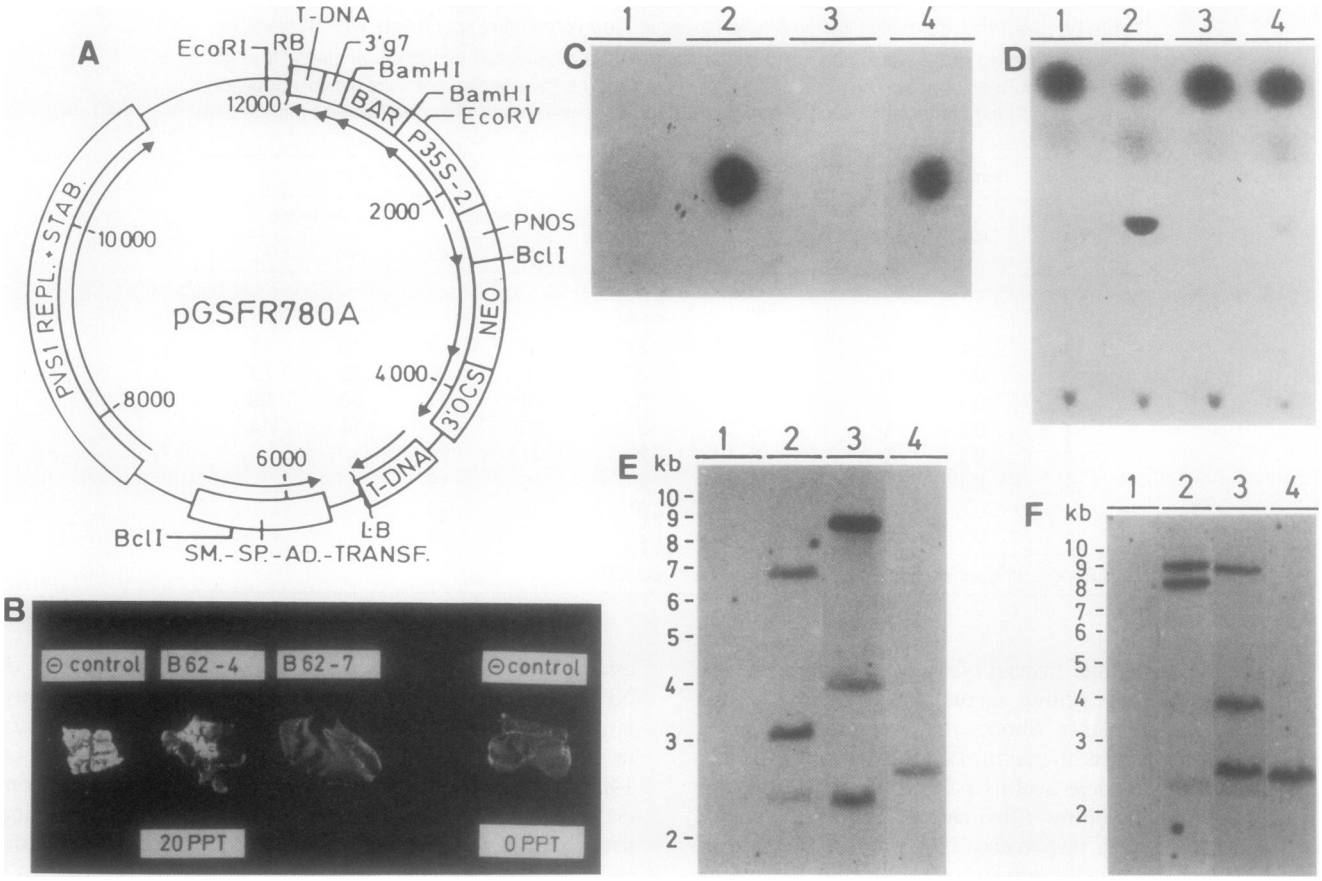

Figure 1. Expression of the *bar* and *neo* genes in transgenic *B. napus* plants, cultivar R8494, transformed with the vector pGSFR780A. A, Schematic representation of pGSFR780A. The *neo* gene is under the control of the nos promoter (12), while the *bar* gene is under the control of the 35S promoter (20). Both genes are inserted between the T-DNA border repeats (*RB*, right border; *LB*, left border). The *neo* and *bar* genes are followed by fragments encoding termination and polyadenylation signals derived from the octopine synthase gene (3'ocs) and the T-DNA gene 7 (3'g7) (26), respectively. B, Leaf pieces of control and transgenic plants were placed on medium A5 containing 20 mg/L phosphinotricin (PPT). The leaf pieces of the most resistant plants stayed green (dark on the picture) and formed healthy callus. Leaf pieces of less resistant plants developed yellow, necrotic regions (light on the picture) but still formed callus. The control leaf pieces turned yellow and never formed callus. C, Detection of the NPTII-activity by the dot assay of McDonnell (16). Dot 1, untransformed control plant; dots 2–4, transgenic plants. D, Detection of the PAT-activity by TLC. Lane 1, untransformed control plant; lanes 2 to 4, three independent transformants expressing the *bar* gene at a different level. The numbers correspond with the numbers of the transformants in C. E, Detection of the *bar* gene. The plant DNA was digested with *EcoRV* and hybridized with a ^{32}P-labeled purified *Bam*HI fragment containing the *bar* gene. Lane 1, untransformed control plant; lanes 2 to 4, transgenic plants. The numbers correspond with the numbers of the transformants in C and D. F, Detection of the *neo* gene. The plant DNA was digested with *Bcl*I and hybridized with a ^{32}P-labeled purified fragment containing the *neo* gene. The lanes correspond with these in E.

ment containing the coding sequence of the *neo* or *bar* gene was used as probe (see "Materials and Methods"). From the 26 characterized *B. napus* transformants, 8 contained one copy; 3, two copies; 11, three copies; and 4 contained more than three copies (up to eight copies) of the whole T-DNA. From the 12 studied *B. oleracea* transformants, 7 contained one copy; 1, two copies; and 4, three copies of the whole T-DNA (Fig. 2). In both *B. napus* and *B. oleracea* there was no correlation between the copy number and the resistance level. The copy number was also not influenced if the selection was done for kanamycin or phosphinotricin resistance.

As described above, some plants selected for kanamycin or phosphinotricin resistance were negative in both the PAT or NPTII enzyme assays. However, partial resistance in the leaf disc assays to both kanamycin and phosphinotricin indicated that these plants had to contain both the *neo* and *bar* genes. Southern hybridizations were done with total DNA of 3 plants selected on 20 mg/L phosphinotricin (Table V) and 10 plants selected on 50 mg/L kanamycin-SO_4, all of which had no detectable PAT and NPTII activities. The southern hybridizations showed (Fig. 1, E and F) that all 13 plants contained one to a few copies of both the *bar* and *neo* genes.

Inheritance of the Chimeric Genes

The transgenic plants were selfed, and the seeds were harvested. To test for the segregation of the *neo* gene, hypocotyl explants or leaf discs of the S1-seedlings were placed on A5-medium with 50 mg/L kanamycin-SO_4. For the segregation of the *bar* gene the 6-week-old S1-seedlings were sprayed with doses equivalent to 8 L Basta/ha, or for those with a low

Table V. *Expression of the bar and neo Genes in B. napus Plants Transformed with the Plasmid pGSFR780A*

Plant No.	Enzyme Activity		Copies *bar* Gene	Resistance in Leaf Disc Assay		Treatment with 8 L Basta/ha		
	PAT	NPTII		PPT[a]	K_m[b]	Resistance	After 6 h	After 24 h
							μg NH_4^+-N/g fr wt	
B62-1	Weak	Weak	NT[c]	+++	+++	RS[d]	173	137
B62-2	N[e]	N	3	++	++	RSS[f]	76	230
B62-3	Strong	Strong	NT	++++	++++	R[g]	22	22
B62-4	N	N	4	++	++	RSS	133	284
B62-7	Strong	Strong	1	++++	++++	R	39	9
B62-8	Strong	Strong	NT	++++	++++	R	36	29
B62-10	N	N	4	+	+	S[h]	191	479
Control	N	N	None	−	−	S	176	440

[a] 20 mg/L phosphinotricin. [b] 50 mg/L kanamycin-SO_4. [c] Not tested. [d] Damaged. [e] Not detectable. [f] Severely damaged. [g] Resistant. [h] Sensitive.

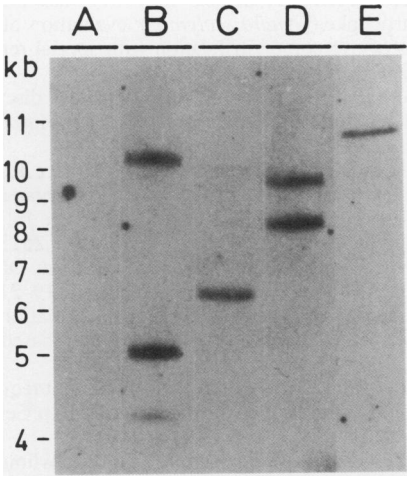

Figure 2. Southern blot analysis of four transgenic *B. oleracea* plants, cultivar Walcheria, transformed with the vector pGSFR780A. The plant DNA was digested with *Eco*RI and hybridized a first time with a ^{32}P-labeled purified fragment containing the *bar* gene. After autoradiography and removing of the probe, the filter was hybridized a second time with a ^{32}P-labeled fragment containing the *neo* gene. The two autoradiograms were identical, indicating that the T-DNA copies contain both the *bar* and *neo* genes. Lane A, untransformed control plant; lanes B to E, transgenic plants.

resistance level, leaf disc assays on medium with 20 mg/L phosphinotricin were done. As expected, normal mendelian segregation rates, which correlated with the copy number of the gene, were found.

DISCUSSION

An efficient and largely genotype-independent transformation system for *Brassica napus* and *Brassica oleracea*, which gives rooted transformed plants in 9 to 12 weeks, is described.

The use of $AgNO_3$ in the medium is a prerequisite. Without $AgNO_3$ none or only very few transformed shoots, depending on the genotype, could be obtained. The stimulating effect of Ag^+ on shoot regeneration has been described (6, 22). For a more detailed description for the use of $AgNO_3$ in the medium and its property as an antiethylene agent in tissue culture, see De Block (6). The most important points are: (a) $AgNO_3$ must be added to the medium after autoclaving; (b) the addition of carbenicillin (250–500 mg/L) prevents the medium from turning brown and avoids toxic effects of Ag^+-ions; (c) $AgNO_3$ cannot be replaced by $Ag_2S_2O_3$. As is described for potato (6), $Ag_2S_2O_3$, although very effective on shoot regeneration, gives toxic effects upon prolonged use.

The concentration of $AgNO_3$ to be added to the medium must be optimized for each genotype. Different concentrations of $AgNO_3$ (we routinely use 2, 5, and 10 mg/L) should be tested after infection and under selective conditions. For example, the *B. oleracea* varieties we tested regenerated better under nonselective conditions if no Ag^+ was added to the medium. However, to get transformed shoots under selective conditions 2 to 5 mg/L $AgNO_3$ had to be added. It may be that meristematic cells present in the hypocotyl explants are not very susceptible to *Agrobacterium* infection, while other cells, which do not regenerate under normal conditions, are transformed. The use of $AgNO_3$ can stimulate these cells to regenerate.

The Ag^+ should be used as early as possible, when selection starts. Once a certain type of nonregenerating callus is formed, it is difficult to reverse this process to shoot regeneration. Probably, the use of Ag^+ in *Brassica* tissue culture can be generalized. For example, preliminary experiments with the *Brassica campestris* cultivar Tobin, which is very difficult to regenerate, indicated that 25 mg/L $AgNO_3$ stimulated shoot regeneration from transformed callus.

Depending on the variety, vitrification posed a problem in the *Brassica* tissue culture. Vitrified shoots are difficult to root and often die shortly after their transfer to the greenhouse. The vitrification of plants *in vitro* is mostly explained by a low concentration of agar and a high relative humidity (2, 5), the latter seeming to be the most important factor. Vitrification can be prevented more effectively by reducing the RH than by decreasing the water potential of the medium (by using higher agar, sucrose, or mannitol concentrations) (2). Also, in the *Brassica* tissue culture, both the RH and the matrix potential of the medium seemed to have an important

influence on the vitrification level. The use of a high agarose concentration (0.5%) and the sealing of the Petri dishes with Urgo pore tape, instead of parafilm, decreased vitrification. Urgo pore tape, which is very permeable, creates a low relative humidity and prevents an accumulation of ethylene (6) and CO_2. We also noticed that in comparison with small and low Petri dishes, the use of big (14 cm diameter) and high (2.2 cm) Petri dishes lowers vitrification (probably also due to a lower RH). Once the shoots are well developed it is important to transfer them as soon as possible to rooting medium A8 with vermiculite.

The proportion of 200 mL vermiculite to 100 mL of liquid medium gives a rather dry, well-aerated medium in which the plants root easily, and produce many root hairs. The shoots that were vitrified often returned (new grown parts) on this medium to a normal, nonvitrified state. We also found that a lowering of the sucrose concentration or using glucose instead of sucrose decreased vitrification. A possible explanation is that a stimulation of chloroplast development counteracts vitrification. Lower levels of sucrose (13) or the use of carbon sources such as glucose induce Chl accumulation, accompanied by further chloroplast development (11, 25).

It is also advisable to prevent a temperature gradient within the culture vessels. A temperature gradient can be created when the bottom is warmed up (when the culture vessels stand above a light source) and the top is cooled down (by a cold air stream). This creates a high RH, which increases vitrification (2).

Besides a high RH and a high water potential of the medium, vitrification can also be increased by high concentrations of cytokinins (1). We noticed that there was much less vitrification when the BAP concentration was dropped as soon as meristems or small shoots were visible on the callus. The low concentration of 0.01 μM BAP was sufficient but necessary to permit the meristems and small shoots to grow out. The complete omission of BAP caused rapid death of the tissue and shoots. As long as the shoots were smaller than 2 cm, 0.01 μM BAP had to be used to prevent senescence.

Selection for kanamycin resistance gave shoots more quickly than if phosphinotricin resistance was used as selectable marker. To check if the plants are transformed, it is important to use the leaf disc assay, which is more sensitive than the NPTII and PAT assays. We found that plants selected for kanamycin or phosphinotricin resistance were often (up to 25%) negative in both enzyme assays. However, these plants had a clear, although weaker, resistant phenotype in both the kanamycin and phosphinotricin leaf disc assays. Southern blottings proved that these plants had not escaped selection but were transformed and contained both the *neo* and *bar* genes. The high frequency of *Brassica* transformants which express the chimeric genes poorly was not observed in tobacco, potato, or tomato (6, 7). Moreover, these low expression levels were found with all the transformation vectors that were used expressing the *neo* or *bar* genes under the control of the nos, TR (26), the *Arabidopsis* rbcS (4, 15) or 35S promoters (data not shown). The reason for this is not understood. Methylation and developmental regulation of the genes are possible explanations. Further experiments in this area are currently underway.

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