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Agrobacterium-mediated transformation and plant regeneration of *Brassica oleracea* var. *capitata*

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Abstract An efficient protocol for *Agrobacterium tumefaciens*-mediated transformation of four commercial cultivars of *Brassica oleracea* var. *capitata* is described. A strain of *A. tumefaciens* LBA4404 with the neomycin phosphotransferase gene (*nptII*) and a CaMV 35S-peroxidase gene cassette were used for cocultivation. Preliminary selection of regenerated transgenic plants was performed on kanamycin-containing medium. The frequency of transgenic plants was calculated on the basis of GUS (β-glucuronidase) activity detected by the histochemical X-gluc test. Tissue-specific GUS expression driven by the peroxidase gene promoter in transgenic plants was analysed by GUS staining. The transformation rates of the commercial cultivars of *B. oleracea* was higher than in previous reports. Southern blot analysis revealed that integration of marker genes occurred in single and multiple loci in the genome. All transgenic plants grew normally after a brief vernalization period and showed stable inheritance of the marker gene. The present study demonstrates that morphologically normal, fertile transgenic plants of *B. oleracea* can be obtained.

Key words *Agrobacterium tumefaciens* · NPT II genes · β-Glucuronidase · Transgenic plants

Introduction

There have been numerous problems in producing cabbage with high yield and of good quality, mainly due to biotic and abiotic factors. In South Africa, the infection caused by TuMV, soft rot, *Peronospora parasitica* and unfavourable environmental conditions (Channon 1981; Lucas et al. 1988) contribute to decrease in yield and quality. Plant breeders in the past several decades have used interspecific sexual hybridization to transfer genes between species. Sexual incompatibility barriers severely limit the possibilities for gene transfer between species, although some of the *Brassica* species can be easily crossed sexually (Puddephat et al. 1996). Specific traits can be introduced into related cultivated crops by genetic transformation techniques (Tinland 1996). The transfer of foreign genes into plants has opened new ways to study regulation of development: it has also provided new approaches to certain breeding objectives of economically important plants (Bent 1996; Kazun et al. 1997). However, a major obstacle in successful genetic transformation in cabbage is the lack of wide availability of genotypes with high capacity for plant regeneration.

Therefore, the objectives of the present study were to improve and establish effective protocols for plant regeneration with high genetic stability and genetic transformation systems in *Brassica oleracea* var. *capitata*, using several genotypes.

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Materials and methods

Plant material and culture media

Seeds of cabbage (*Brassica oleracea* var. *capitata*) cultivars Hercules, Brunswick, Cape spitz and Copenhagen were surface sterilized with ethanol (1 min), 0.1% $HgCl_2$ (10 min), commercial sodium hypochloride (0.4% active chlorine, 20 min), and thoroughly rinsed with sterile water (Neskovic et al. 1987). Seeds were allowed to germinate on half-strength Murashige and Skoog medium (1962). Cotyledons were aseptically removed and transferred to culture medium as described by Boulter et al. (1990). The constituents of the basal medium were similar to those described earlier (Palmer 1992) with minor changes. It comprised B_5 mineral salts (Gamborg et al. 1968), 3% sucrose, 0.7% agar and (in ml l^{-1}): thiamine 0.4, pyridoxine 0.5, nicotinic acid 0.5, *m*-inositol 100 and casein hydrolysate 2000. The basal medium was supplemented with 2.2 mg l^{-1} benzyladenine (BA)

and 0.17 mgl^{-1} indoleacetic acid (IAA) to induce organogenesis. Medium without any growth regulators served as the control.

Vectors and bacterial strains

Two binary vectors containing T-DNA border sequences and the *nptII* gene as the plant selective marker were used. The first vector (pKK6) contained *Agrobacterium tumefaciens* with the oncogenic strain A281, which harbours the plasmid pTiBo542 in the avirulent A136 cells (Montoya et al. 1977) and A281 containing the binary vector pGA472, with the GUS gene and pTiBo542 as the helper plasmid (An et al. 1985) were used. The tumour tissue induced by oncogenic A348 (Garfinkel et al. 1981) and Ach5 (De Vos et al. 1981) strains were used for comparison in opine and DNA analysis. *A. tumefaciens* was maintained on agar (1.5%) solidified yeast extract (Van Larebeke et al. 1977), supplemented with rifamycin (5 μmg ml^{-1}) and nalidixic acid (50 μmg ml^{-1}) for A281 and A348 strains, or kanamycin (Km; 10 μmg ml^{-1}) and rifamycin (3 μmg ml^{-1}) for pGA472. For inoculation, bacteria were grown overnight in liquid medium in shake cultures at 30 °C, to about 5×10^8 cells per ml^{-1}.

Transformation and regeneration

Inoculation with bacteria was performed using a modified leaf disk method (Horsch et al. 1985). The excised cotyledonary fragments were placed into bacterial suspension for a few minutes and blotted by transferring the cotyledons to the media supplemented with 1 gl^{-1} carbenicillin. The antibiotic was retained at a lower concentration (0.5 gl^{-1}) for three to four further subcultures.

When the cotyledons were inoculated with oncogenic strains, a basal hormone-free medium was used for co-cultivation and culture of resulting calli. However, the established regeneration protocol (Srejovic and Neskovic 1981) was followed for inducing organogenesis. Briefly, cotyledon fragments were pre-cultured for 5 days on the cell division medium. They were inoculated with A281/pGA472 suspension and subsequently cultured either on the regeneration medium, or on tobacco nurse culture, according to Rogers et al. (1986). Control explants were treated similarly, except that they were incubated in sterile Yeast Extract Broth medium. Bacterial growth was suppressed with carbenicillin.

When shoots were regenerated on cotyledonary fragments, they were propagated on regeneration medium for two passages, and then subjected to Km selection, from 0–250 μmgml^{-1} of KM. In another experiment, cotyledonary fragments were put on the selection medium immediately after incubation with bacteria.

Evidence of transformation – Southern blot and GUS analysis

Transformation with oncogenic strains was confirmed by opine analysis and DNA hybridization tests (Herrman et al. 1986). One gram fresh weight of uncloned A348 and Ach5 tumour tissue, grown aseptically on hormone-free medium for 2 months, was cultured overnight in arginine (10 m*M*)-enriched medium, homogenized and analysed according to Lichtenstein and Draper (1985). Extracts of A281 tumour tissue were analysed for the presence of mannopine (David et al. 1984). The *Eco*RI fragment 7 (7.3 kb) from pTiB6S3 (De Vos et al. 1981) clones in pBR325 carried by *Escherichia coli* HB 101 was used as the DNA probe.

To confirm transformation of regenerated plants, the NPTII activity assay and DNA hybridization tests were performed. Crude extracts of 0.5–1.0 g fresh tissue, which was macerated in the extraction buffer (An et al. 1985), was processed as described by Radke et al. (1988). DNA was extracted from transformed regenerated plantlets and controls (Dellaporta et al. 1985) digested with *Bam*HI and *Hin*dIII enzymes and Southern hybridization was performed according to Herrman et al. (1986). It was labeled using a DIG High Prime DNA Labeling and Detection Kit (colour detection with NBT/BCIP; Boehringer Mannheim, Germany). *Hin*dIII-linearized pGA472 was isolated from *E. coli* (Maniatis et al. 1982).

Results and discussion

Regeneration

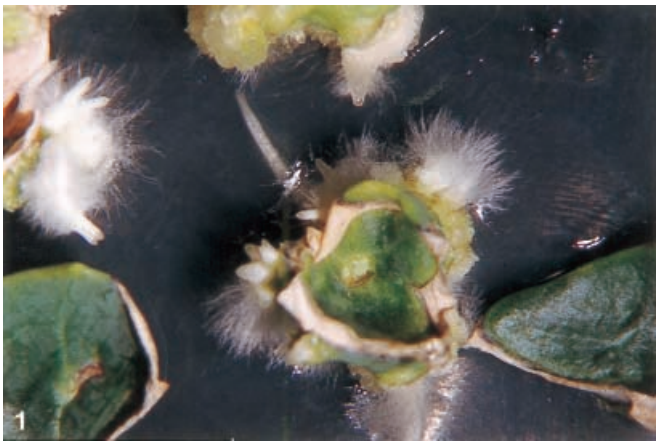
Transformation experiments with A281/pGA472 were performed with 80 cotyledon pairs, while 20 were used as controls. In both groups the regeneration capacity was 32% (Table 1), which was within the range as reported in buckwheat by Srejovic and Neskovic (1981). The rest of the explants predominantly produced callus tissue with roots. Regenerated buds from the controls and putative transformed explants were multiplied and cloned during two passages. Shoot tips with apices about 2 mm long were excised from each clone and placed in petri dishes containing 10 ml of regeneration medium, supplemented with Km in various concentrations. The apices of untransformed plants become necrotic at concentrations higher than 10 μg ml^{-1}. The assay with regenerants from A281/pGA472-incubated cotyledons showed that in all the clones tested, all the buds were sensitive and shrivelled even at low concentrations of Km (Fig. 1). The green buds in the surviving clones were cultured for seven passages on 100 μmgml^{-1} of Km. Under selection pressure clones of resistant buds were established.

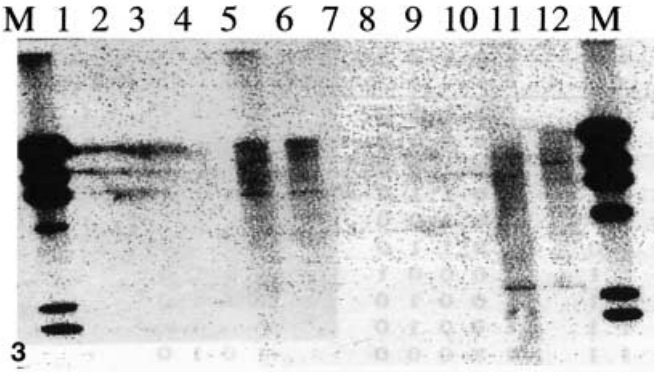
Preculture of cotyledons on a high concentration of 2,4-dichlorophenoxyacetic acid induced cell division in a large number of cells (Neskovic et al. 1985). Prolonged selection in Km-containing medium is required, since the regenerated buds have a muticellular origin. When the cotyledons were transferred immediately to the selection medium, resistance was observed in 14.3% of explants, since regeneration occurred only in transformed plants. The regenerated shoots appeared

Table 1 Bud regeneration and screening of transformants in *Brassica oleracea* (*Km* kanamycin)

Cotyledon pairs	Control	Incubated with A281/pGA472
Expt 1[a]		
No. of cotyledon pairs	20	80
With regenerated buds	7 (32%)	25 (31.25%)
Km-resistant clones	0	14 (56%)
Expt. 2[b]		
No. of cotyledon pairs	10	70
Km-resistant	0	10 (14.3%)
with regenerated buds	–	4 (40%)
undifferentiated calli	–	6 (60%)

[a] Bud cultures tested for Km resistance in third subculture
[b] Explants transferred to Km (100 μmg ml^{-1}) containing medium immediately upon incubation with A281/pGA472





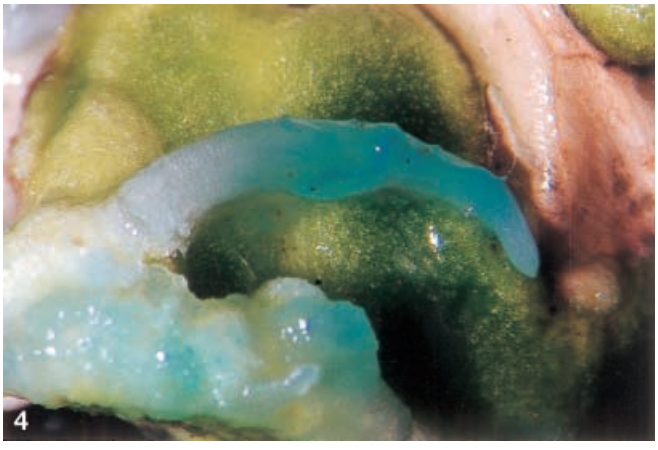


Fig. 1 Selection of clones on kanamycin Km (2 μm gml^{-1}). Note some clones are transformed, showing root induction and other non-transformed show shrivelled margins

Fig. 2 Transformed plant acclimatized in greenhouse conditions

Fig. 3 Southern blot of kanamycin/hygromycin resistant calli to a blot of genomic DNA digested with *Eco*RI probed with shpx6 cDNA. M Marker (λ *Hin*dIII digest), *lanes 1,2, 3, 5,6,11,12* transformed calli, *lanes 8,9,10* non-transformed calli, *lanes 4,7* control

Fig. 4 Transformed calli stained with X-gluc to reveal GUS activity, 3 weeks after *Agrobacterium* transformation. Note that the stronger expression in the primary root reflects the pattern of expression typical of CaMV 35 S promoter constructs

morphologically similar (Fig. 2). This confirms the finding of An et al. (1986) that the use of wild type Ti plasmid B0542 as helper does not impair the regeneration of Km-resistant shoots. Transformed plantlets were raised by rooting the shoots on filter paper bridges over liquid medium containing 0.2 mgl^{-1} IBA.

Transformation

Plants were initially screened for evidence of transformation by determining callus formation from leaf discs of transgenic shoots. Tissues from non-transgenic control plants turned white or brown and showed no expression. Transformation was confirmed by Southern hybridization, using the shpx6cDNA clone as the hybridization probe. Most of the lines tested had multiple T-DNA insertions (Table 2). Hybridization was not detected with untransformed control plants (Fig. 3). Other workers have observed similar phenomena in transformation of canola and related species (Moloney et al. 1989). Multiple insertion during *Agrobacterium*-mediated gene transfer has been sug-

Table 2 Number of T-DNA loci and number of integrated T-DNA copies for the pSDM transformed lines

T-DNA loci[a]	T-DNA inserts[b]	Lines
1	1	12
	2	3
	3	1
	4	1
2	2	2
	3	1
	3	0
3	4	0
	5	2

[a] No. of segregating T-DNA loci calculated from the ratio of hygromycin-resistant versus sensitive seedlings on hygromycin-selective medium using χ^2 analysis

[b] Actual no. of independent T-DNA inserts calculated from the combined Southern blot for 22 lines tested

Table 3 Genetic analysis of the progeny from five transgenic plants for the kanamycin resistance trait

Plant no.	Km^R progeny	Km^S progeny	Ratio	X^2	No. of loci
2	99	9	11:1	0.80	2
4	111	9	13:1	0.32	2
5	96	15	6:1	0.12*	1–2
6	30	7	3:1	0.73	1
7	90	7	12:1	0.15	2

* $P < 0.05$

gested to be a feature of *Brassica*ceae (Fry et al. 1987). However, it may also be dependent on the selection schemes and the levels or types of antibodies used, as suggested by Moloney et al. (1989). Almost all the plants tested had copies of the transgene. Most of these plants were morphologically indistinguishable from the control plants grown under similar conditions. Genetic inheritance of the transgene has been determined by scoring the progeny of five lines of transgenic plants for tolerance to Km. The transgenics had both Km^R and Km^S plants. χ^2 analysis confirmed that most of them segregated for at least two functional T-DNA copies (Table 3).

Transformation with oncogenic strains

The cotyledonary fragments incubated with A281 and other oncogenic strains produced callus tissues on cut surfaces, which were grown axenically on hormone-free media for several months (Neskovic et al. 1990). Octopine was present in A348 and Ach5 calli and mannopine in A281-transformed calli, while these were not present in the controls. The hybridization of genomic DNA from tumours, with a DNA probe, confirmed that the oncogenic *A. tumefaciens* strains brought about stable genetic transformation of *Brassica* tissues. The DNA of the A281 strain tumour contained two fragments in *Bam*HI digest and three fragments in the *Hin*dIII digest which hybridized with the probe. No hybridization occurred in DNA digests of untransformed callus tissue. The size of the fragments obtained from digested Ach5 tumour tissue DNA and from Ach5 DNA corresponded to those obtained in the restriction map by De Vos et al. (1981). The digested DNA from Ach tumour contained more hybridizing bands than those present in T-DNA of pTiAch5.

Histochemical-GUS gene expression under the control of the 5′ sequences from a gene encoding a peroxidase enzyme in the pasture legume *Subtilus humilis* was introduced into the cultivars and regenerates were analysed by GUS staining of the tissue. Analysis of five transgenic plants by GUS staining indicated constitutive GUS expression on the young root initials (Fig. 4). Wound inducible expression was also evident on the petioles and stems and this expression pattern is consistent with the reports of Kazun et al. (1997).

Conclusion

In the present study, a hypocotyl and a root transformation method for *Brassica oleracea* have been employed. Although transformation efficiencies are not very high with the cultivars used, it is still easy to generate reasonably large numbers of transgenic plants. The transformation methods described here are reproducible and transformation of these lines should produce genetically uniform plants differing only at transgene loci for the evaluation of the effects of introduced genes on the physiological and agronomical performance of *Brassica* plants. Based on these preliminary results, introduction of other genes into the *Brassica* genotypes for assessment of enhanced disease resistance is under progress.

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