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Transformation of *Brassica napus* via *Agrobacterium* Vector : Plant Regeneration and Progeny Analysis

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Agrobacterium 운반체를 이용한 유채의 형질전환: 식물체재분화와 후대검정

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Cotyledonary petioles of *Brassica napus* cocultivated with *Agrobacterium* vectors for 72 h were transferred to MS medium with 0.5 mg/L NAA, 2.0 mg/L BA, 30 mg/L kanamycin, 100 mg/L cefotaxime, 30 g/L sucrose, 3 mg/L $AgNO_3$ and 2 g/L Gelrite. The cotyledonary petioles with green shoots were selected at a frequency of 17.5% in a selection medium and then rooted. Southern blot analysis confirmed the *rolC* and *NPT Ⅱ* genes were incorporated into the regenerated plants. The stable inheritance of *rolC* gene was confirmed in progeny test of transgenic plants.

Key words: *Brassica napus*, *Agrobacterium*, transgenic plant.

Recently several reports concerning the transformation of plants using foreign gene have been made. In this field, the *Cruciferae* including some *Brassica* spp. are excellent host plants for bacterial vector systems (De Block et al., 1989). Transformation methods of the genus *Brassica* include cocultivation of *B. napus* with *Agrobacterium* vector (Charest et al., 1988), microinjection of DNA into cell (Neuhaus et al., 1987), and the electroporation performed in plant protoplasts (Chapel and Glimelius, 1990). In the transformation study of a plant which uses *Agrobacterium* vector, it is important that the frequency of plant regeneration is enhanced by explants co-cultivated with *Agrobacterium*. A variety of tissues such as stem explants (Pua et al., 1987), hypocotyl (De Block et al., 1989), and cotyledons (Moloney et al., 1989) of *B. napus*, have been used to improve the transformation efficiency. As a result, the use of *B. napus* as a model plant for transformation studies has not been efficiently improved.

The present paper reports an appropriate methodology for in vitro culture of *B. napus* and the development of an efficient procedure for genetic transformation via co-cultivation of cotyledons with *Agrobacterium* vector of *rolC* which was associated with dwarfism of plant (Oono et al., 1990). Analysis of transformants, T_1 and T_2 plants was performed through biochemical assay for the *rolC* gene activity (NPT Ⅱ), cotyledon culture on selective medium, and Southern blot analysis.

MATERIALS AND METHODS

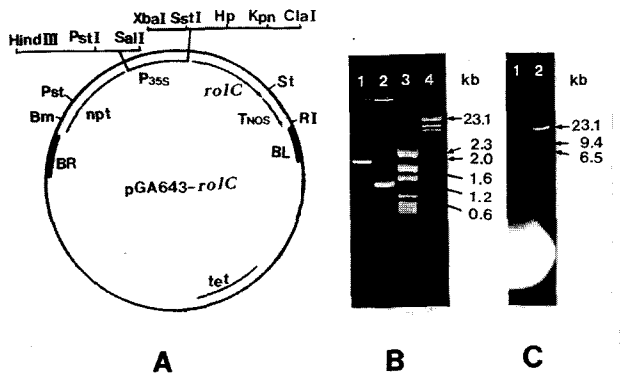
Plant Materials and *Agrobacterium* Vector

Seeds of *Brassica napus* cv. Naehanyuche were kindly supplied by National Yeongnam Agriculture Experiment Station, Korea. Seeds were treated for 12 h at 4°C, and then sterilized in 70% ethanol for 30 sec, and 1% sodium hypochlorite for 30 min. They were washed in sterile distilled water three times and inoculated at a density of 25 seeds per plate(5×12cm) on 20 mL Murashige-Skoog (MS, 1962) medium with 3% sucrose and 0.2% Gelrite. The pH was

adjusted to 5.8 before autoclaving. Seeds were germinated at 26°C in a 16 h light/8 h dark photoperiod at light intesity of 60~80 $\mu Em^{-2}s^{-1}$. *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) containing a binary vector was used. The binary vector, pGA 643-*rolC* (Figure 1) carries the neomycin phosphotransferase Ⅱ gene (*npt* Ⅱ). *Agrobacterium* was grown overnight at 28°C in the darkness at 120 rpm in LB medium (1% Bacto-peptone, 0.5% Bacto-yeast extract, 1% NaCl) supplemented with kanamycin(km, 50 mg/L) and carbenicilin (25 mg/L).

Transformation of *B. napus* via *Agrobacterium tumefaciens*

Cotyledons were excised at 5 d after seed germination that they included 2 mm petioles at the base. Care was taken to eliminate the apical meristem which sometimes adheres to the petioles. Individual excised cotyledons were taken from the plants described above and the cut surface of their petioles was immersed into this bacterial suspension for 2~4 s. They were immediately placed on the MS medium, 3% sucrose and 0.2% Gelrite enriched with 0.5 mg/L α-naphtalenacetic acid (NAA) and 0.2 mg/L 6-benzyladenine (BA). The cotyledons were co-cultivated with the *Agrobacterium* for 72 h. After co-cultivation, the cotyledons were transferred to regeneration medium including MS medium supplemented with 0.5 mg/L NAA, 2.0 mg/L BA, 100 mg/L cefotaxime, 30 mg/L kanamycin, 3 mg/L $AgNO_3$. Each plate contained 10 explants. The explants were cultured on regeneration medium under light and temperature conditions specified above, for 30 d and then formations of root and shoot were investigated.



Figiure 1. A: Structure of the expression vector. p35s, CaMV 35s promotor: *rolC*, coding region of the ORF 12 gene of *Agrobacterium rhizogenes*: Tnos, nopaline synthase terminator: BR, T-DNA right border: BL, T-DNA left border: npt, chimeric nos-npt (nopaline synthase-neomycin phosphotransferase) that serves as a selectable marker in plants: Tet, tetracycline resistance gene. B: *rolC* fragment from pGA 643-*rolC* vector. Lane 1: eluted *npt* Ⅱ/*Bam*H Ⅰ-*Hind* Ⅲ, Lane 2: eluted *rolC*/*Eco*R Ⅰ-*Hpa* Ⅰ, Lane 3: pGEM size maker, Lane 4: λ digested with *Hind* Ⅲ. C: Identification of pGA 643-*rolC*. Lane 1: intact pGA 643-*rolC*, Lane 2: λ digested with *Hind* Ⅲ.

Southern Blot Analysis

Regenerated plantlets in MS medium with the appropriate antibiotics were transferred to 'rooting' medium containing MS medium, 3% sucrose, 0.5 mg/L 3-indoleacetic acid (IAA), and 0.2% Gelrite. As soon as a small root mass was obtained, the plantlets were transferred to potting mix supplemented with vermiculite:perlites:peatmoss(1:1:1). The plants were transferred to field.

Leaf tissue (approximately 1 g) from plants 1~2 weeks old was ground to a powder in liquid nitrogen and DNA was isolated according to the method described by Dellaporta et al. (1983). For Southern blot analysis, DNA was digested with *Eco*R Ⅰ-*Hpa* Ⅰ and resolved by electrophoresis on a 0.8% agarose gel in a 0.5X TBE buffer (0.04417M Tris, 0.044M Boric acid, 1 μM EDTA). Resolved DNA was transferred to a nylon membrane (Hybond-N, Amersham) through standard methods as described by Southern (1975). The probes were randomly labelled with ^{32}P(an Amersham kit) to a specific activity 1 × 10^9 cpm/μg. Hybridization to probes was performed in 0.5 M NaH_2PO_4 (pH 7.2), 1% BSA, 1 mM EDTA and 7% SDS at 65°C. The membranes were washed twice in 2X SSC, 0.1% SDS for 15 min at room temperature and then twice in 0.1X SSC, 0.1% SDS for 20 min at 42°C. Then filters were exposed to Kodak XAR-5 film using Dupont Cronex Quanta B intensifier screens at -80°C.

Genetic Analysis of Transgenic Progeny

Seeds from self-pollinated transformed plants and from a control plant were treated for 12 h at 4°C, and then were sterilized as described above. The seed were inoculated at a density 30 seeds per plate (5×12 cm) on 20 mL MS medium supplemented with 3% sucrose, 30 mg/L kanamycin, and 0.2% Gelrite. Seeds were cultured at 26°C in 16 h light/8 h dark photoperiod light intensity of 60~80 $\mu Em^{-2}s^{-1}$. After 40 d, resistance or sensitivity was scored according to the presence/absence of normal roots and shoot growth. The Chi-square test was used for testing hypothesis concerning a different number of T-DNA insertions, and a different possible transmission of kanamycin resistance to the progeny.

RESULTS AND DISCUSSION

The addition of $AgNO_3$ in the medium enhanced remarkably the shoot regeneration from the cotyledonary petioles. The maximum frequency of shoot regeneration (74.2%) was obtained from the cotyledonary petioles cultured on the medium with 3 mg/L $AgNO_3$ (Table 1). A comparison of different procedures for regeneration of *Brassica* spp. indicates wide variations in regeneration efficiency and measured by yield of differentiated shoots obtained. The regeneration efficiency varies between 67% for cotyledon and 42% for hypocotyl explants (Chi et al., 1990). The yield of regenerated plants obtained by this procedure was higher than in Chi et al.(1990).

Table 1. Effect of $AgNO_3$ on plant regeneration from cotyledonary petioles of *B. napus*

$AgNO_3$	No. of cotyledons		
(mg/L)	cultured	shooting (%)	rooting (%)
0	135	27(20.0)	65(48.1)
3	155	115(74.2)	132(85.2)

In transformation via co-cultivation of cotyledons with *Agrobacterium* vetor of *rolC* gene, shoot regeneration frequencies for co-cultivation was 17.5% (Table 2). A comparison of different procedures for transformation of *B. napus* indicates wide variations in transformation efficiency as measured by yield of transformed shoots obtained. The transformation efficiency varies between 2% for epidermal explants (Charest et al., 1988), or 2.5% for hypocotyl explants (Radke et al., 1988) and 10% for stem explants (Pua et al., 1987). The yield of transformed plants obtained by this procedure was higher than in these three papers. But the transformation efficiency of this study was not higher than that of Molony et al. (1989). It may be attribute to different genotype of donor plants tested.

The green shoots regenerated on medium with antibiotics were recultured in the same medium with antibiotics (Figure 2). Southern blot showed in Figure 3. Genomic DNAs of transgenic plants (lanes 1~6) and control (lane 7) were digested with *Eco*R I -*Hpa* I restriction enzyme and probed with the *rolC* and *npt*Ⅱ gene. In the results, questionable transformed plants were detected a 2.0kb band (*rolC* gene) in the lane 4. It is presumed that tranformed plants were detected a 1.0 kb and a 2.0 kb band (*npt*Ⅱ and *rolC*, respectively) in the lanes 2 and 6.

Table 2. Frequency of shoot regeneration from cotyledon of *B. napus* cocultivated with *Agrobacterium* vector

No. of cotyledons	No. of cotyledons forming shoots(%)		
cultured	total	green	white
120	54(45.0)	21(17.5)	33(27.5)

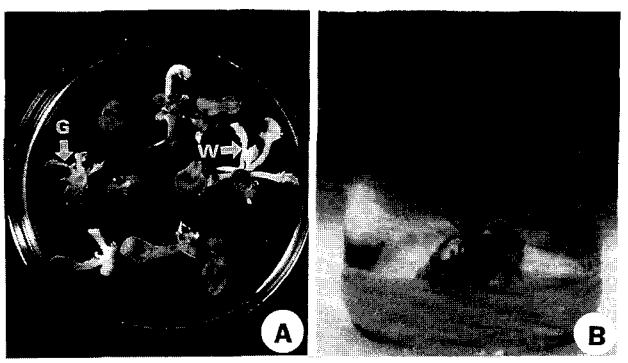


Figure 2. Shoot formation from cotyledonary petioles cocultivated with *Agrobacterium* vectors in *B. napus*. A: Shoot formation from cotyledonary petioles on selection medium (W: white. G: green). B: Normal green plant reselected from the medium with kanamycin.

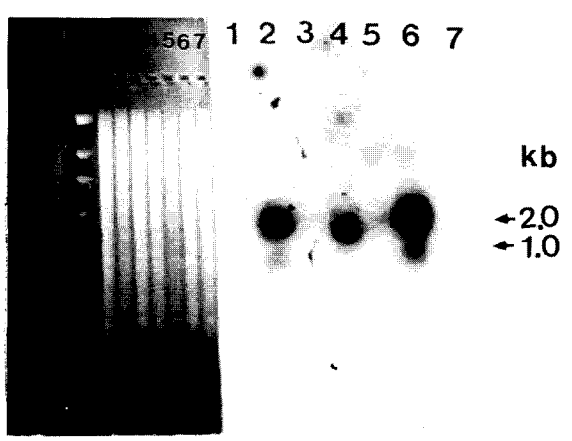


Figure 3. Southern blot analysis of *Eco*R I -*Hpa* I digests of genomic DNA isolated from putative transformed *B. napus*. Probe DNA: 1kb of *rolC* coding sequence and 2kb of *npt*Ⅱ coding sequence. Lanes 1~6: samples, 7: control, 8: λ DNA (*Hind*Ⅲ digested).

The seeds of individual transformed plants were germinated normally in selective conditions (30 mg/L kanamycin) while nontransformed progenies did not. The segregation ratio for km resistance of seedlings derived from self-fertilization of three independent transgenic plants were analyzed and results are reported in Table 3. The transformants were segregated

Table 3. Segregation ratio for kanamycin resistance of seedlings derived from self-fertilization of three independent transgenic plants (km^R=kanamycin resistance; km^S=km sensitivity).

T_2 family	Segregation of		x^2 values (p=0.05)
	km^R	km^S	3:1
T_2-2	84	31	0.23
T_2-4	58	11	3.02
T_2-6	91	21	2.33
Control	0	114	-

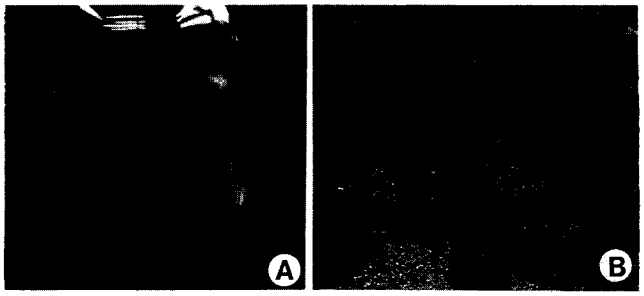


Figure 4. Transgenic *B. napus* cv. Naehanyuche. A: Plants from T_2 seeds on selection medium (R: resistance, S: sensitivity). B: Transformants grown in soil.

with ratio of 3 km resistance:1 km sensitivity.

Rotino et al. (1992) reported that 9 lines segregated according to the Mendelian Genetics from 11 lines of transformed Solanum integrifolium via *Agrobacterium tumefaciens* in km resistance examination. In this experiment, T-DNA of putative transformed plants with *rolC* gene was inherited in progeny.

적 요

유채의 자엽조직을 *rolC* 유전자를 가진 *Agrobacterium* 운반체와 공동배양하여 형질전환체를 얻고, 후대에 대한 유전분석을 실시하였다. *Agrobacterium* 운반체와 공동배양된 유채의 자엽조직을 5 mg/L NAA, 2.0 mg/L BA, 30 mg/L kanamycin, 100 mg/L cefotaxime, 30 g/L sucrose, 3 mg/L $AgNO_3$ 및 2 g/L Gelrite가 첨가된 MS 배지에 배양하였을 때 형질전환된 식물체의 분화율이 17.5%로 나타났다. 형질전환체로 확인된 식물체를 자가수정시켜 얻어진 후대에 대한 유전분석을 실시한 바 *rolC* 유전자가 후대로 유전됨을 확인 할 수 있었다.

REFERENCES

Chapel M, Glimelius K (1990) Temporary inhibition of cell wall synthesis improves the transient expression of the GUS gene in *Brassica napus* mesophyll protoplasts. Plant Cell Reports **9**: 105-108

Charest PJ, Holbrook LA, Gabard J, Iyer VN, Miki BL (1988) *Agrobacterium*-mediated transformation of thin cell layer explants from *Brassica napus* L.. Theor Appl Genet **75**: 438-445

Chi GL, Barfield DG, Sim GE, Pua EC (1990) Effect of $AgNO_3$ and aminoethoxyvinylglycine on in vitro shoot and root organogenesis from seedling explants of recalcitrant *Brassica* genotypes. Plant Cell Reports **9**: 195-198.

De Block M, Brouwer DD, Tenning P (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of the bar and neo genes in the transgenic plants. Plant Physiol **91**: 694-701

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA mini-preparation : version Ⅱ. Plant Mole Biol Reporter **4**: 19-21

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

Moloney MM, Walker JM, Sharma KK (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Reports **8**: 238-242

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant **15**: 473-477

Neuhaus G, Spangenberg G, Scheid OM, Schweiger HG (1987) Transgenic rapeseed plants obtained by the microinjection of DNA into microspore-derived embryoids. Theor Appl Genet **75**: 30-36

Oono Y, Kanaya K, Uchimiya H (1990) Early flowering in transgenic tobacco plants possessing the *rolC* gene of *Agrobacterium rhizogenes* Ri plasmid. Jpn J Genet **65**: 7-16

Pua EC, Mehra-Palta A, Nagy F, Chua NH (1987) Transgenic plants of *Brassica napus* L.. Bio/Thechnology **5**: 815-817

Radke SE, Andrews BM, Moloney MM, Crouch ML, Kridl JC, Knauf VC (1988) Transformation of *Brassica napus* L. using *Agrobacterium tumefaciens*: developmentally regulated expression of a reintroduced napin gene. Theor Appl Genet **75**: 685-694

Rotino GL, Perrone D, Ajmone-Marsan P, Lupotto E (1992) Transformation of *Solanum integrifolium* Poir via *Agrobacterium tumefaciens*: Plant regeneration and progeny analysis. Plant Cell Reports **11**: 11-15

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol **98**: 505-517

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