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High-Frequency Regeneration and Transformation of *Raphanus sativus*

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We have achieved high-frequency shoot regeneration in radish (*Raphanus sativus*). Cotyledon explants from four-day-old seedlings were suitable for the effective induction of shoots on Murashige and Skoog's (MS) medium containing 3.0 mg/L kinetin. We also determined that it was essential to include 1- to 2-mm petiole segments with the cotyledons for efficient induction. When the regenerated shoots were transferred to an MS liquid medium containing 0.1 mg/L NAA, roots formed within four weeks, and normal plant development ensued. We established a transformation protocol using an *Agrobacterium* binary vector that carries the GUS reporter gene. Preculturing the explants for 1 d in an MS medium containing 3.0 mg/L kinetin also increased efficiency. Five days of cocultivation proved best for delivering T-DNA into radish. Transformation frequencies of up to 52% were obtained in shoot induction media that contained 3.0 mg/L kinetin.

Keywords: *Agrobacterium*, GUS, plant regeneration, radish (*Raphanus sativus*), transformation

Regeneration of mature plants from undifferentiated tissues is central to the application of biotechnology for crop improvement. Successful transformation systems require efficient and reliable regeneration protocols for introducing available foreign genes into plants. The radish (*Raphanus sativus*) has been regenerated via organogenesis (Park et al., 1996), somatic embryogenesis (Jeong et al., 1995; Takahata et al., 1996), and micropropagation (Paek et al., 1987). In these studies, radish hypocotyl segments produced calli on an MS medium containing 2,4-D. In addition, Jeong et al. (1995) showed that up to 5.3% of the calli gave rise to somatic embryos upon transfer to a medium containing BA and NAA. Approximately 90% of those embryos developed into plantlets on half-strength MS basal media.

Shoots have been regenerated directly from hypocotyl explants of Chinese radish (*R. sativus* L. var. *longipinnatus*; Pua et al., 1996). Matsubara and Hegazi (1990) studied callus initiation and plantlet regeneration for eight cultivars of *R. sativus*, and found that the frequency of shoot and plantlet regeneration was below 7% from three of those cultivars, 'Comet', 'Moriguchi', and 'Nerima Shirinaga'. Park et al. (1996) noted that hypocotyl explants of 'Taewang' formed calli on an MS medium containing 2,4-D, kinetin, and BA; they also observed normal shoot regeneration on the medium carrying IAA and kinetin.

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Although *Agrobacterium*-mediated T-DNA transformation has been reported for *Brassicaceae* species (Charest et al., 1988; Radke et al., 1988; Moloney et al., 1989), transformation of *Raphanus* has been difficult. Curtis and Nam (2001) demonstrated that transgenic radish plants could be produced by the floral-dip method. In the current study, we introduced a foreign gene into the *R. sativus* genome using *Agrobacterium tumefaciens* LBA4404, which contained the GUS gene (Jefferson, 1987). Our objective was to manipulate simple culture conditions to obtain the most efficient regeneration from excised cotyledons of *R. sativus*.

MATERIALS AND METHODS

Plant Regeneration

We used the *R. sativus* cultivar RS91. After sterilization in a 2% (v/v) sodium hypochlorite solution, seeds were placed on a germination medium comprising Murashige and Skoog (1962) salts and vitamins (MS), 3% sucrose, and 0.7% agar, and were maintained at 25 ± 2°C under a 16-h photoperiod. We then excised the cotyledons, including their 1- to -2-mm petioles, from four-day-old seedlings. The petioles were embedded in an MS basal medium supplemented with a combination of either 0.0 to 1.0 mg/L IAA and 0.0 to 5.0 mg/L kinetin, or 0.0 to 1.0 mg/L NAA and 0.0 to 5.0 mg/L BA. Ten explants were placed on each Petri

plate. After three weeks of culture, we counted the adventitious shoots and roots that formed on the explants. Regeneration frequency was estimated by averaging four plates, with a score of 'one' assigned to an explant even when multiple regenerants were produced. The regenerated shoots were then transferred to MS, 1/2-MS, and 1/4-MS media for rooting. After four weeks, regenerated plants with well-developed roots were acclimatized in water for 7 d, then transferred to soil.

Transformation

For transformation, we used the binary Ti-plasmid vector plG121-Hm (Ohta et al., 1990). This vector contains an intron within the GUS gene. *A. tumefaciens* LBA4404 (for carrying the transformation vector) was grown for 24 h at 28°C (250 rpm) in a YEP medium containing 250 mg/L streptomycin, 50 mg/L kanamycin, and 50 mg/L hygromycin, to an optical density of 0.8 at 600 nm. Cotyledons from four-day-old seedlings were cultured for 1 d in an MS medium (pH 5.7) containing 3.0 mg/L kinetin, 3% sucrose, and 0.7% agar. These precultured cotyledonary explants were submerged for 5 min in an inoculum of LBA4404, then cocultured for 5 d in the same media type. Afterward, the explants were transferred to an MS medium (pH 5.7) that contained 3% sucrose, 0.7% agar, 0.1 mg/L kinetin, 0.1 mg/L NAA, 15 mg/L kanamycin, and 250 mg/L claforan. Shoots were induced after three weeks.

We used the procedure of Jefferson (1987) to histochemically determine GUS activity in the regenerated plants. Shoots were washed with sterile deionized water and incubated at 37°C for 24 h in a 3-mL GUS solution containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.1 M sodium phosphate buffer (pH 7.0), 0.1% X-Gluc, 0.1% Triton X-100, and 10 mM EDTA. The tissues were soaked in 75% ethanol for 24 h to remove the chlorophyll.

RESULTS AND DISCUSSION

Optimization of Plant Regeneration

Of the various hormone combinations tested, we found that 3.0 mg/L kinetin (KIN) was the best media supplement for promoting shoot formation (Table 1). In fact, both IAA and NAA inhibited shoot regeneration. Shoots formed within 1- to 2 mm of the petiole end having contact with the medium (Fig. 1, A and B).

Table 1. Effects of auxin and cytokinin concentrations on shoot and root regeneration from cotyledon explants.

Auxin (mg/L)	Cytokinin (mg/L)		Shoots (%)	Roots (%)
IAA 0.0	KIN	0.0	30.0	40.0
		1.0	58.8	2.5
		3.0	72.5	0.0
		5.0	52.5	0.0
IAA 0.1	KIN	0.0	17.5	60.0
		1.0	40.0	2.5
		3.0	27.5	0.0
		5.0	42.5	0.0
IAA 1.0	KIN	0.0	5.0	77.5
		1.0	10.0	0.0
		3.0	30.0	2.5
		5.0	20.0	2.5
NAA 0.0	BA	0.0	30.0	40.0
		1.0	76.7	0.0
		3.0	45.0	0.0
		5.0	50.0	0.0
NAA 0.1	BA	0.0	17.5	62.5
		1.0	57.5	5.0
		3.0	45.0	2.5
		5.0	37.5	0.0
NAA 1.0	BA	0.0	5.0	65.0
		1.0	7.5	7.5
		3.0	0.0	0.0
		5.0	0.0	0.0

These shoots were directly regenerated from explants containing vascular tissues. This type of regeneration has also been reported in *Pinus radiata* (Biondi and Thorpe, 1982), *Iris hollandica* (Hussey, 1976), and *Allium sativum* (Moriconi et al., 1989). In contrast, aberrant shoots formed in the BA- and NAA-supplemented media. Root regeneration was enhanced by increasing the concentration of auxin, but was strongly inhibited by the cytokinin (Table 1).

These results are consistent with that of Kim (1997), who found that shoots of 'Hannong' radish were regenerated on a medium supplemented with IAA and kinetin, but not with NAA and BA. Park et al. (1996) also reported that shoots of 'Taewang' radish developed on 2.0 mg/L IAA and 7.0 mg/L kinetin. In a study by Matsubara and Hegazi (1990), radish explants formed calli on a medium containing 2,4-D and BA, with kinetin being more effective than BA for shoot induction. Our results confirm those previous findings that IAA plus kinetin is the best hormone combination for radish regeneration.

To increase root induction efficiency, we examined the effect of MS salt concentrations and agar (Fig. 2), and found that liquid media were more effective than

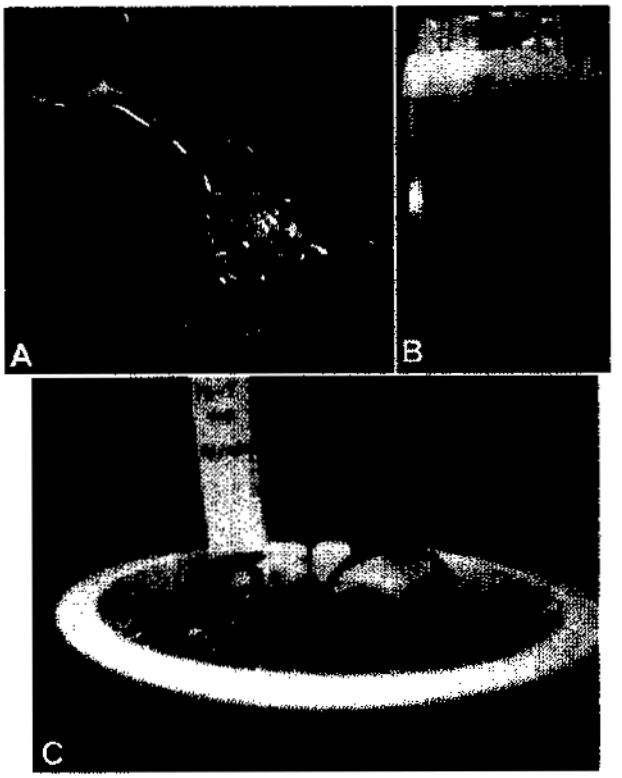

Figure 1. A. Shoot regeneration from cotyledon explant of *R. sativus* cv. RS91 on MS medium containing 1.0 mg/L kinetin under light. pe, petiole; sh, shoot; Bar = 1 mm. **B.** Regenerated plantlet of radish transferred to MS medium. **C.** Regen-

solid media. However, salt concentration was not a controlling factor in root formation. Whereas root development on solid media took 20 d, only 10 d were needed on the liquid media. Roots that regenerated on solid media were thin and long, and were easily loosened during acclimatization. In contrast, roots regenerated in liquid media were thicker and healthy. In addition, the frequency of root formation increased to 99% when 0.1 mg/L NAA was included in the liquid media. When regenerated plantlets were transferred to soil, they showed normal growth (Fig. 1C).

In another experiment (data not shown), we observed that adding $AgNO_3$ or using gas-permeable MicroPore surgical tape (3M Co., St. Paul, MN, USA) for sealing our Petri dishes did not influence shoot-induction frequency. This result contradicts that of Pua et al. (1996), who showed that the exogenous application of an ethylene inhibitor (aminoethoxyvinylglycine) or $AgNO_3$ enhanced shoot regeneration of hypocotyl explants derived from Chinese radish. We also suggest that one critical factor determining shoot regeneration is the quality of the meristematic cells in explants. For exam-

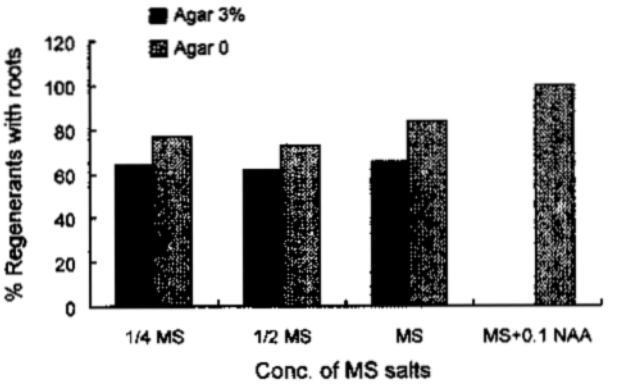

Figure 2. Effects of the concentration of MS salts and agar on root regeneration. Explants were cultured at 25°C for four weeks under 16-h photoperiod.

ple, in mangosteen (*Garcinia mangostana*), the width of the lamina remaining on either side of the cotyledon midrib influenced shoot differentiation (Goh et al., 1994).

Transformation of Radish

We used *Agrobacterium*-mediated transformation to develop a procedure for introducing a foreign gene into radish. To identify the optimum concentration of kanamycin for transformant selection, we examined the basal levels of kanamycin resistance in radish. From our cotyledonary explants, two to three white shoots were occasionally induced on media carrying 10- to 20 mg/L kanamycin, but they eventually turned black (data not shown). No shoots were formed at the higher concentrations. Therefore, we believe that 15 mg/L kanamycin is adequate for selection of transformants.

In our experiment on the effects of growth regulators on transient expression of the GUS reporter gene, we found that cytokinin is essential for radish transformation (Table 2). We also observed that preculturing

Table 2. Effects of growth regulators on transient GUS expression after cocultivation.

Growth regulator (mg/L)	Cocultivated explants	Explants with shoots	Shoots expressing GUS	Transformation frequency (%)
none	50	0	0	0
BA (1)	50	31	19	38
KIN (1)	50	15	5	10
KIN (3)	50	37	26	52
NAA (0.1)	50	5	0	0

Transformation frequency = GUS-expressing shoots/cocultivated explants.

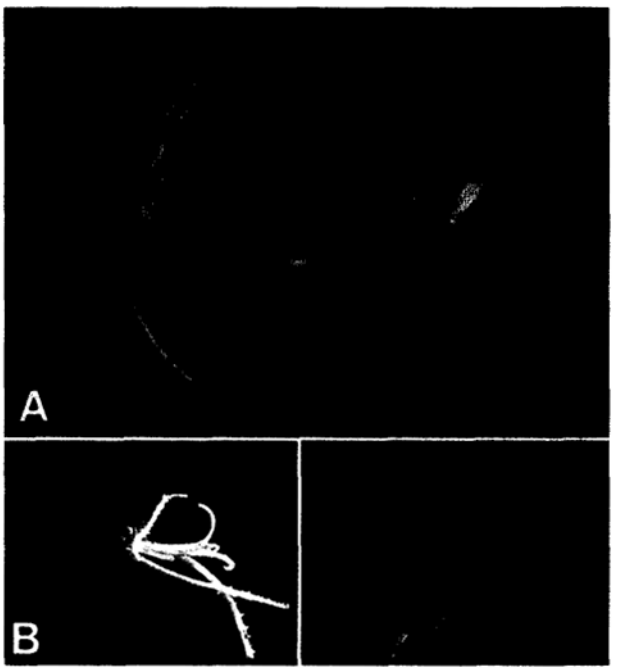

Figure 3. Selection of transformed shoots and plantlets. The cotyledonary petiole explants were cocultivated with *A. tumefaciens* LBA4404 carrying the binary plasmid pIG121-Hm. **A.** Shoot differentiation in the presence of kanamycin. **B.** Non-resistance shoots became chlorotic (left). Survivor (right) developed roots in the presence of NAA. pe, petiole; sh, shoot; rt, root.

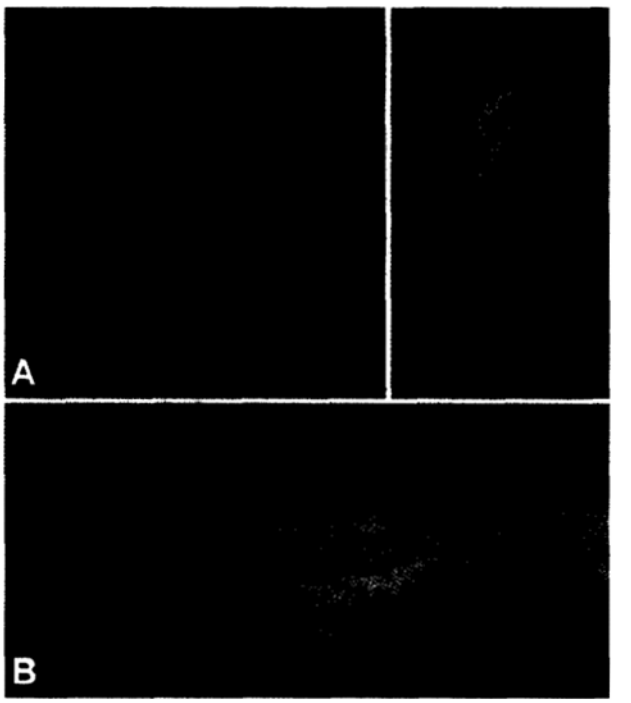

Figure 4. Histochemical GUS assay of transgenic shoots. **A.** GUS assay of transgenic shoots 2 weeks (left) and 3 weeks (right) after cocultivation. pe, petiole; sh, shoot. **B.** GUS assay of a leaf from a transgenic plant.

the explants for 1 d in a cytokinin-containing medium enhanced transformation frequency. Indeed, GUS expression was minimal from explants grown on the medium containing no hormones or one supplemented only with auxin.

Another critical factor that influences transformation efficiency is the length of the cocultivation period with *Agrobacterium*. Radke et al. (1992) suggested that plant cell division is induced by cocultivation stress. We also observed that 5 d of cocultivation promoted higher frequencies than did shorter periods (data not shown). Our histochemical assay showed that GUS was strongly expressed at the cut ends of the cotyledonary explants, an observation consistent with previous studies (Eapen and George, 1994; Voisey et al., 1994; Arokiaraj et al., 1998).

After cocultivation, the explants were cultured for two weeks on a medium containing 0.1 mg/L NAA, 0.1 mg/L kinetin, 15 mg/L kanamycin, and 250 mg/L claforan. Shoots formed on the cut ends of these cotyledonary explants (Fig. 3). Because our histochemical GUS assay of the shoots confirmed expression of the reporter gene, we assert that the regenerants are, indeed, transgenic plants (Fig. 4).

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LITERATURE CITED

Arokiaraj P, Yeang HY, Cheong KF, Hamzah S, Jones H, Coomber S, Charlwood BV (1998) CaMV 35S promoter directs β-glucuronidase expression in the laticiferous system of transgenic *Hevea brasiliensis*. Plant Cell Rep 17: 621-625

Biondi S, Thorpe TA (1982) Growth regulator effects, metabolite changes, and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata*. Bot Gaz 143: 20-25

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

Curtis IS, Nam HG (2001) Transgenic radish (*Raphanus*

sativus L. *longipinnatus* Bailey) by floral-dip method-plant development and surfactant are important in optimizing transformation efficiency. Transgen Res 10: 363-371

Eapen S, George L (1994) *Agrobacterium tumefaciens* mediated gene transfer in peanut (*Arachis hypogaea* L.). Plant Cell Rep 13: 582-586

Goh CJ, Lakshmanan P, Loh CS (1994) High frequency direct shoot bud regeneration from excised leaves of mangosteen (*Garcinia mangostana* L.) Plant Sci 101: 173-180

Hussey G (1976) Propagation of Dutch iris by tissue culture. Scient Hortic 4: 163-165

Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol Biol Rep 5: 387-405

Jeong WJ, Min SR, Liu JR (1995) Somatic embryogenesis and plant regeneration in tissue culture of radish (*Raphanus sativus* L.). Plant Cell Rep 14: 648-651

Kim NR (1997) Plant regeneration via callus culture from hypocotyl explants and organogenesis from cotyledon explants of radish (*Raphanus sativus*). Master thesis. The Catholic University of Korea, Puchon

Matsubara S, Hegazi HH (1990) Plant regeneration from hypocotyl callus of radish. HortScience 25: 1286-1288

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

Moriconi DN, Conci VC, Nome SF (1989) In vitro plantlet regeneration from callus in garlic (*Allium sativum* L.). Φyton 49: 97-103

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-479

Ohta S, Mita S, Hattori T, Nakamura K (1990) Construction and expression in tobacco of β-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. Plant Cell Physiol 31: 805-813

Paek KY, Chandler SF, Thorpe TA (1987) Micropropagation of *Raphanus sativus* L. var. *longipinnatus* (Japanese radish) cv. Gungjung. Plant Cell Tiss Org Cult 9: 159-165

Park MC, Son SI, Kim JC (1996) Plant regeneration from hypocotyl-derived callus of radish (*Raphanus sativus*). Kor J Plant Tiss Cult 23: 243-247

Pua EC, Sim GE, Chi GL, Kong LF (1996) Synergistic effect of ethylene inhibitors and putrescine on shoot regeneration from hypocotyl explants of Chinese radish (*Raphanus sativus* L. var. *longipinnatus* Bailey) *in vitro*. Plant Cell Rep 15: 685-690

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

Radke SE, Turner JC, Facciotti D (1992) Transformation and regeneration of *Brassica rapa* using *Agrobacterium tumefaciens*. Plant Cell Rep 11: 499-505

Takahata Y, Komatsu H, Kaizuma N (1996) Microspore culture of radish (*Raphanus sativus* L.):I Influence of genotype and culture conditions on embryogenesis. Plant Cell Rep 16: 163-166

Voisey CR, White D, Dudas B, Appleby TD, Ealing PM, Scott AG (1994) *Agrobacterium*-mediated transformation of white clover using direct shoot organogenesis. Plant Cell Rep 13: 309-314