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Agrobacterium-Mediated Transformation System for Large-Scale Producion of Transgenic Chinese Cabbage (*Brassica rapa* L. ssp. *pekinensis*) Plants for Insertional Mutagenesis

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In order to better utilize insertional mutagenesis and functional genomics in Chinese cabbage, we have developed an improved transformation system that more efficiently produces a large number of transgenic plants. Hypocotyl explants were inoculated with *Agrobacterium tumefaciens* LBA4404. This strain harbors tagging vector pRCV2, which contains a hygromycin-resistance gene, an ampicillin resistance gene, and a bacterial replication origin within the T-DNA. Transformation efficiency was highest when the explants were first co-cultivated for 3 d in a medium supplemented with 5 mg L^{-1} acetosyringone, then transferred to a 0.8% agar selection medium containing 10 mg L^{-1} hygromycin. In addition, maintaining a low pH in the co-cultivation medium was critical to enhancing transformation frequency. A total of 3369 transgenic plants were obtained, with efficiencies ranging from 2.89% to 5.00%. Southern blot analysis and T_1 progeny tests from 120 transgenic plants confirmed that the transgenes were stably inherited to the next generation. We also conducted plasmid rescue and inverse PCR with some transformants, based on their phenotype, to demonstrate the applicability of T-DNA tagging in Chinese cabbage. The tagged sequences were then analyzed.

Keywords: acetosyringone, *Agrobacterium tumefaciens*, genetic transformation, T-DNA tagging

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*), one of the most important vegetables for agricultural production, is widely cultivated in Asia, especially in Korea, Japan, and China. Genetic engineering techniques, such as *Agrobacterium*-mediated DNA transfer, have great potential for improving established cultivars by introducing genes of interest while maintaining commercially desirable phenotypes. Moreover, the application of insertional mutagenesis using *Agrobacterium* T-DNA is an attractive approach for functional genomics in which the number of steps required for conceptually linking a given gene to its function minimized (Spradling et al., 1995). Insertional mutagenesis has become a valuable tool for the identification and isolation of genes now that researchers have developed several strategies for screening T-DNA in a known gene and recovering the sequences that flank the insertions (Koes et al., 1995; Liu and Whittier, 1995; Cooley et al., 1996; Frey et al., 1998; Couteau et al., 1999; Krysan et al., 1999). The T-DNA of *Agrobacterium* can be used for saturating the genome with insertional mutations in order to establish correlations among sequence data, mutant phenotypes, and gene functions via reverse genetics (Feldmann et al., 1994).

Because Chinese cabbage is in the same taxonomic family as *Arabidopsis thaliana* and shares more than 80% homology in gene structure, data from the *Arabidopsis* genome sequencing project is useful in the study of gene functioning in Chinese cabbage. However, to apply the *Agrobacterium*-mediated DNA transfer technique to *Braccica rapa*, a large number of transgenic plants as well as high transformation efficiency are necessary.

Several reports on its *Agrobacterium*-mediated transformation have now demonstrated that Chinese cabbage is no longer considered impossible to engineer (Mukhopadhyay et al., 1992; Jun et al., 1995; Christey et al., 1997; Takasaki et al., 1997; Zhang et al., 2000; Cho et al., 2001). In particular, the use of acetosyringone (AS) in the bacterial culturing and co-cultivation media is most critical when cotyledonary

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explants are used to regenerate shoots (Zhang et al., 1998, 2000). Moreover, hygromycin-selection of transformants is effective in minimizing the proportion of 'escapes' when transforming with hypocotyl explants (Cho et al., 2001).

Despite these successes, no procedure has yet been reported for producing a sufficiently large number of transgenic plants in Chinese cabbage. This has been due to two factor: the inability to decrease the rate of 'escapes' that develop under kanamycin selection in systems using cotyledonary explants, and the difficulty in increasing transformation efficiency when using hypocotyl explants. Therefore, the objectives of this study were to: (1) optimize the conditions needed for stably producing an adequate number of transgenic plants and (2) improve transformation efficiency in two Korean cultivars of Chinese cabbage. Furthermore, we investigated the utility of T-DNA tagging in Chinese cabbage by applying plasmid rescue and inverse PCR to some of our transformants created with *Agrobacterium tumefaciens* LBA4404 harboring the tagging vector pRCV2.

MATERIALS AND METHODS

Transformation Vector and *Agrobacterium* Strain

Chinese cabbage (*B. rapa* L. ssp. *pekinensis*) was transformed with the T-DNA tagging vector pRCV2 (Kim et al., 2003), which contains the hygromycin phosphotransferase (*hpt*) gene for hygromycin resistance and the ampicillin-resistant gene for rescue of plant flanking DNA (Fig. 1). This vector was constructed by ligating pCAMBIA 1301 with pBluescript II KS(+) (Stratagene, USA), similar to the method for creating pRCV3 (Kim et al., 2003). Disarmed *A. tumefaciens* strain LBA 4404 served as a helper strain in the binary vector system. After the T-DNA tagging vector pRCV2 was introduced via the freeze-thaw method (An et al., 1988), Agrobacteria were grown in a YEP medium prior to transformation.

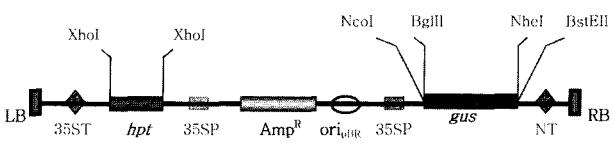

Figure 1. T-DNA region of binary vector pRCV2. LB, left border; RB, right border; 35ST, 3' signal of CaMV 35S; *hpt*, hygromycin resistance gene; 35SP, CaMV 35S promoter; Amp^R, ampicillin-resistance gene active in *E. coli*; NT, 3' signal of nopaline synthase; ORI, origin of replication of ColE1.

Plant Material and Preparation

Two cultivars of Chinese cabbage, 'Seoul' (F1 hybrid; Dong-bu Seed, Korea) and 'DA001' (inbred line; Dong-bu) were used for the transformation experiments. Seeds first were submerged in 70% ethanol for 1 min, then in 30% commercial Clorox (1.6% hypochlorite) plus 0.1% Tween-20 for 20 min, with vigorous shaking. They were rinsed three times, germinated under lights on an MS medium (Murashige and Skoog, 1962), and held for 7 d under a 16 h photoperiod until the hypocotyls were 4~5 cm long. The hypocotyls were then dissected, avoiding the shoot apex, and quickly cut into 7- to 10-mm segments.

Transformation and Selection Procedures

To determine the optimum conditions for improved transformation efficiency, 1 ml of aliquot from our *Agrobacterium* cell stock was cultured in 50 ml of a YEP medium containing 50 mg L^{-1} kanamycin and 5 mg L^{-1} AS (acetosyringone), to an OD_{600} value of 1.0. The *Agrobacterium* cultures were pelleted, washed, and re-suspended in 50 ml of the YEP medium. Hypocotyl explants were then infected by immersing them in the bacterial inoculum for 10 min. Afterward, to test the effectiveness of AS and media pH, the explants were placed on sterile filter paper and embedded in a co-cultivation medium with or without 5 mg L^{-1} AS and at either pH 5.2 or 5.7. After 3 d of co-cultivation, the explants were washed with an MS liquid medium supplemented with 200 mg L^{-1} cefotaxime, and transferred to an MS selection medium containing 3% sucrose, 4 mg L^{-1} BA, 3 mg L^{-1} NAA, 4 mg L^{-1} $AgNO_3$, 5 mg L^{-1} AS, 200 mg L^{-1} cefotaxime, 10 mg L^{-1} hygromycin, and 0.8% plant agar (pH 5.6). After cultivation for 3 and 8 weeks, we determined the number of calli and shoots, respectively, that formed on the explants. Finally, the regenerated shoots were transferred to a rooting medium that consisted of 1/2-strength MS medium, 3% sucrose, 200 mg L^{-1} cefotaxime, and 0.7% plant agar (pH 5.8).

PCR Confirmation of Transformation

To detect the *hpt* and *gus* genes in the genome of our transformed plants, 50 ng of genomic DNA was used for PCR amplification with the *hpt* and *gus* primer sets, which were designed to yield either a 700-bp internal *hpt* fragment or a 600-bp internal *gus* fragment. The amplifications were performed in a 50 μl reaction mixture with *Taq* polymerase and a thermocy-

cler (Biometra, USA). Each of 35 PCR cycles comprised 1 min-denaturation at 95°C and 1 min-annealing at 55°C, followed by a 2 min-extension at 72°C.

Southern Hybridization

We digested 10 μg of genomic DNA with *Xho*I or *Bam*HI, separated it by electrophoresis on a 0.9% agarose gel, and transferred it to a Hybond N^+ membrane (Amersham Pharmacia Biotech, USA). The separated DNA was probed with a ^{32}P-labeled 700 bp *Xho*I fragment of the *hpt* gene from pRCV plasmid DNA to confirm integration of the T-DNA into the plant genome. Random primer labeling of the probe was conducted according to the manufacturer's protocol for the Takara Ladderman TM Labeling Kit (Takara, Japan).

Progeny Test

One hundred twenty transgenic plants were vernalized at 4°C for 40 d, and then each was self-pollinated. To analyze the segregation ratio, we germinated 40 sterilized seeds from each line on selection media containing 20 ml L^{-1} hygromycin. After 3 weeks, the number of resistant and susceptible seedlings was counted.

Rescue Cloning and Inverse PCR

Plant genomic DNA was isolated from 17 transgenic lines carrying the T-DNA of the pRCV2 vector. Twenty micrograms of genomic DNA was digested with *Pst*I, *Bam*HI, or *Hin*dIII; each sample was then, ligated and extracted by phenol-chloroform. One half of the circularized DNA was used to transform electroporation-competent cells (Epicurian Coli SURE, Stratagene) in a Bio-Rad *Escherichia coli* Pulser Unit (Bio-Rad, USA) according to the supplier's instructions. The other half was used for inverse PCR, which was performed in a 50 μl reaction mixture with *EF-Taq* polymerase (Solgent, Korea) and a Biometra thermocycler. The 35 PCR cycles each comprised 1 min-denaturation at 95°C, 1 min-annealing at 60°C and a 2 min-extension at 72°C

Sequence Analysis

The purified plasmid from our rescue clones was sequenced using an adjacent primer for flanking plant DNA. The inverse-PCR products were inserted in a pGEM-T vector (Promega, USA) and automatically sequenced with T7 and SP6 primers at the Macrogen (Korea). Flanking sequences were subjected to BLAST searches at the National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

Improvement of Transformation Efficiency and Production of a Large Number of Transgenic Plants from Chinese Cabbage

To analyze the effect of acetosyringone and media pH on transformation efficiency, we evaluated the number of calli and shoots regenerated from infected explants under various co-cultivation conditions (Table 1). Optimum results were achieved when hypocotyl explants were reared in a co-cultivation medium containing 5 mg L^{-1} AS, at pH 5.2. In fact the addition of AS nearly doubled the rate of calli formation. The number of regenerated shoots also was remarkably increased under this protocol.

Using the optimized conditions described above, we co-cultivated hypocotyl explants of two cultivars,

Table 1. Effect of pH and acetosyringone (AS) of co-cultivation media on callus and shoot formation in Chinese cabbage (*B. rapa* L. ssp. *pekinensis*) cv. Seoul.

Co-cultivation medium		No. of explant examined	No. of explant with calli	Ratio of callus formation (%)[z]	No. of shoot regenerated
pH	AS				
5.7	-[y]	2500	470	18.80	39
5.7	+	2500	811	32.44	88
5.2	-	2500	525	21.00	47
5.2	+	2500	1026	41.04	124

[z] (No of explant with calli/no. of explant examined) × 100

[y] -: Without supplemental acetosyringone; +: with 5 mg L^{-1} acetosyringone.

'Seoul' and 'DA001', on media supplemented with 4 mg L^{-1} $AgNO_3$, and 5 mg L^{-1} AS, at pH 5.2. Within 2 to 3 weeks of transfer, calli began to form at the cut ends of hypocotyls that had been placed on a selection medium containing 10 mg L^{-1} hygromycin. Shoots then regenerated from most of the calli within 6 weeks, and these were transferred to the rooting medium.

To confirm integration of T-DNA into the Chinese cabbage genome, we isolated genomic DNA from the leaf tissue of transformed plants and analyzed this via PCR and Southern blotting.

Our PCR results indicated the presence of the expected 700-bp and 600-bp products for the *hpt* and *gus* genes, respectively, in most transformed plants (data not shown). However, no PCR signals were detected in the genomic DNA of some transformed lines that were considered 'escapes'. The rates of shoot formation per explant were 5.23% and 3.02% for 'Seoul' and 'DA001', respectively, while the rate for the 'escapes' was 4.3%. Therefore, the transformation efficiencies were 5.0% for 'Seoul' and 2.89% for 'DA001' (Table 2).

Southern blot analysis also revealed the expected 700-bp *hpt* gene in those PCR-confirmed transgenic plants (Fig. 2). We were able to obtain a total of 3369 hygromycin-resistant shoots from our two cultivars, some of which were then rooted in soil (Fig. 3). Among those 120 T_0 lines confirmed by Southern blot analysis, 63 (52.5%) showed one copy of the transgene, 33 had two, and the remainder had three to five copies (Table 3). These T_0 lines were self-polli-

Table 2. Transformation frequencies of Chinese cabbage (*B. rapa* L. ssp. *pekinensis*) cv. Seoul and DA001.

	Seoul	DA001
No. of infected explants	50,000	30,000
No. of explant with calli and formation frequency (%)[z]	20660 (41.32%)	8590 (28.63%)
No. of explant with shoot and induction frequency (%)[y]	2616 (5.23%)	906 (3.02%)
No. of transformed shoot confirmed by PCR	2503	866
Transformation frequency[x]	5.00%	2.89%

[z](No. of infected explant / No. of explant with calli)×100.
[y](No. of infected explant / No. of explant with shoot)×100.
[x](No. of infected explant / No. of transformed shoot confirmed by PCR)×100.

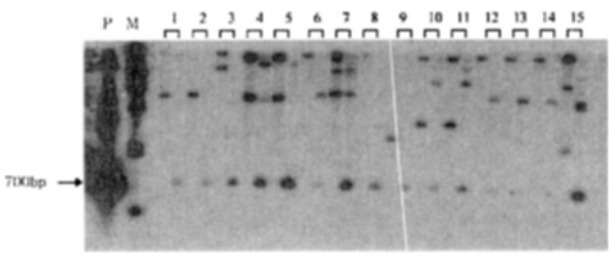

Figure 2. Southern hybridization of DNA from transformants with pRCV2. Genomic DNA was digested with *Bam*HI and *Xho*I, and probed with a ^{32}P-labeled 0.7 kb *Xho*I fragment of *hpt* gene from pRCV2 plasmid DNA. Genomic DNA was digested with *Bam*HI (left) and *Xho*I (right) in the same line, respectively. P, positive marker (RCV2 plasmid); M, molecular marker; 1, T_0 Line 4; 2, T_0 Line 5; 3, T_0 Line 9; 4, T_0 Line 27; 5, T_0 Line 36; 6, T_0 Line 39; 7, T_0 Line 48; 8, T_0 Line 50; 9, T_0 Line 55; 10, T_0 Line 108; 11, T_0 Line 109; 12, T_0 Line 111; 13, T_0 Line 112; 14, T_0 Line 116; 15, T_0 Line 117.

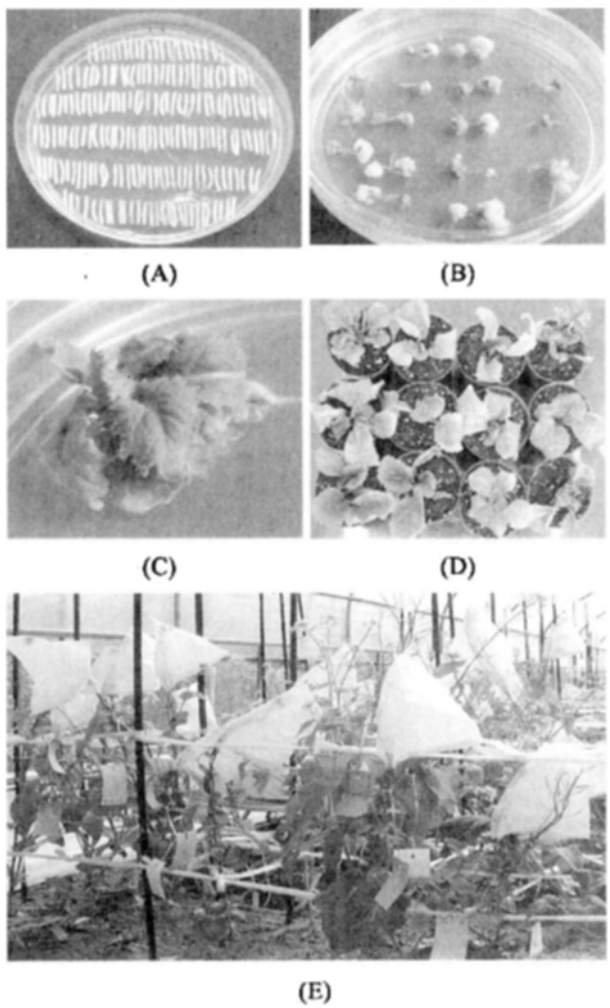

Figure 3. *A. tumefaciens*-mediated transformation of Chinese cabbage cv. 'Seoul'. (a) Co-cultivation; (b) Callus induction; (c) Shoot induction; (d) Acclimation; (e) Self pollination.

nated to obtain T_1 progeny. To examine the segregation ratio in the T_1 generation, 40 seeds from each transgenic line were tested for hygromycin resistance. In all, 63 lines had a progeny segregation ratio of approximately 3:1 for resistance, which is the expected Mendelian inheritance of one independent locus (Table 4). The rest had ratios greater than 3:1. This result nearly corresponded to the copy numbers identifies in our Southern blot analysis, except that five lines (1, 22, 39, 69, and 113) had a segregation ratio of 1:1, i.e., abnormal for resistance (data not shown).

Although in preliminary trials our cotyledonary explants were highly regenerable, transformation success was limited, especially when hygromycin was used as the selection agent in this study (data not shown). Therefore, we chose to transform hypocotyls explants instead. Hygromycin is very effective in decreasing the regeneration of non-transgenic escapes, so we used this agent, at 10 mg L^{-1}, rather than kanamycin, which often allows a higher occurrence of such escapes (Takasaki et al., 1997).

The probability of infection by *Agrobacterium* depends on activation of the *vir* genes in the Ti plasmid. Their induced expression is optimal under acidic conditions, and is positively regulated by phenolic compounds such as AS (Rohini and Rao, 2000). Likewise, in our study, pH of 5.2 for co-cultivation media supplemented with AS enhanced the frequency of *Agrobacterium*-mediated transformation.

Application of T-DNA Tagging in Transgenic Chinese Cabbage

To determine whether T-DNA tagging is useful in Chinese cabbage, we isolated the flanking plant DNA from seven transgenic lines in order to analyze their integrated patterns. The PCR confirmation primer sets used here were capable of amplifying in several

Table 3. Inserted T-DNA copy numbers of T_0 transgenic plants transformed with *A. tumefaciens* LBA4404 harboring pRCV2.

	Inserted T-DNA copy number						Total
	1	2	3	4	5	Not Determine	
No. of transgenic lines	63	33	8	6	5	5	120
Ratio (%)	52.5	27.5	6.6	5.0	4.2	4.2	100

Table 4. Segregation ratios for hygromycin (HPT) resistance and fertility in T_1 selfed-progenies transformed with *A. tumefaciens* LBA4404 harboring pRCV2.

T_0 transgenic line	Fertility[z]	Segregation ratio[y] HPT^R : HPT^S	Expected copy number	X^2 test
4	2.0	37:13	1	1.20
5	3.4	29:11	1	0.13
9	1.2	19:2	2	0.38
27	1.8	43:7	2	0.11
36	0.6	36:4	2	0.96
39	2.2	24:16	-	-
48	2.0	43:2	3	0.11
50	2.7	29:11	1	0.13
55	0.7	29:11	1	0.13
108	3.1	36: 4	2	0.96
109	2.4	37:3	2	0.11
111	2.1	34:6	1	2.13
112	1.1	27:13	1	1.20
116	2.9	31:9	1	0.13
117	4.7	38:2	3	0.11

[z]No. of pollinated flower / no. of seed harvested.
[y]HPT^R, resistant; HPT^S, susceptible.

Table 5. BLAST analysis with sequenced T-DNA tags.

Transgenic plant line	Similarity to	Identity (%)	Reference
1	*Gerbera hybrida* mRNA for dihydroflavonol-4-reductase (DFR)	78	Unpublished
2	Not reacted	-	-
4	Not reacted	-	-
6	*Arabidopsis thaliana* DNA Chromosome 4, contig fragment No. 55	87	Unpublished
7	*Arabidopsis thaliana* DNA Chromosome 5, BAC clone F12B17	83	Unpublished
9	Unknown	-	-
12	*Arabidopsis thaliana* genomic DNA, Chromosome 5, P1 clone	42	Unpublished

important regions of the pRCV2 vector, e.g., the right border backbone. Based on these data, we performed inverse PCR with suitable primers that best adjoined the flanked plant DNA. The inverse-PCR products and the plasmid DNA obtained by this rescue cloning were analyzed, and the sequence information was subjected to BLAST homology searches (Table 5). The flanking plant DNA from Line 1 manifested significant alignments with *Gerbera hybrida* mRNA for dihydroflavonol-4-reductase (DFR); Lines 6, 7 and 12 showed similarity to genes or genomic sequences in *Arabidopsis*. However, we were unable to detect any phenotypic similarity between the Chinese cabbage mutants and *Arabidopsis* mutants in tagged Line 1. Therefore, we continue to investigate the function of those genes disrupted by T-DNA.

The predicted primers confirmed by genomic PCR for Lines 2 and 4 did not react in the inverse PCR, possibly because these transgenic lines had a tandem repeats structure. We are now assessing the function of those disrupted genes. For Lines 9 and 12, we used sequence analysis and genomic PCR to detect unknown fragments inserted to the inside of the T-DNA (data not shown), perhaps during chromosomal recombination. Nevertheless, based on these overall results, we believe that the T-DNA tagging technique is applicable to Chinese cabbage.

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