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Agrobacterium tumefaciens-mediated Hairy Root Production from Seedlings of Chinese Cabbage

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Cruciferous hairy roots are often used for improving drought adaptability, peroxidase production, and *in vitro* subculturing of *Plasmodiophora brassicae*. For metabolic engineering, *Agrobacterium tumefaciens*-mediated systems have previously been developed for hairy root production in other plant species. Here, we used the *rolABC* gene binary construct in *A. tumefaciens* strain GV3101 to establish cultures of Chinese cabbage hairy roots. On both solid and liquid media, the *rolABC* hairy root lines exhibited a wild-type hairy root syndrome in terms of their growth and morphology. This demonstrates that those three genes are sufficient to induce high-quality hairy roots in Chinese cabbage. Such a system could be useful for the stable production of secondary metabolites in that species.

Keywords: *Agrobacterium tumefaciens*, *Brassica rapa* ssp. *pekinensis*, genetic transformation, hairy root

Chinese cabbage (*Brassica rapa* ssp. *pekinensis*), a member of the Cruciferae family (or *Brassicaceae*), is one of the most important vegetable crops in Asia. Cruciferous plants are rich in sulfur-containing compounds known as glucosinolates, many of which have antioxidant and anticancer properties (Jongen, 1996; Talalay and Zhang, 1996). In plants, they play defensive roles such as in disease resistance (Tierens et al., 2001; Brader et al., 2006), the deterrence of generalist herbivores, or the attraction of specialist herbivores (Giamoustaris and Mithen, 1995; Kim and Jander, 2007).

Hairy roots induced by *Agrobacterium rhizogenes* are characterized by their rapid growth of highly branched roots on a phytohormone-free medium (Toivonen and Rosenqvist, 1995; Kittipongpatana et al., 1998). These roots stably produce secondary metabolites over a long period due to their inherent genetic and biochemical stability (Saitou et al., 1991; Agostini et al., 1997). In addition, they are often able to regenerate into whole plants (Hu and Du, 2006). Hairy roots also have been used to study their physiological and morphological differences from non-transgenic roots, drought adaptability, production of peroxidase, and *in vitro* subculture of *Plasmodiophora brassicae*, an obligate parasite that causes cruciferous clubroot disease (Vartanian and Berkaloff, 1989; Agostini et al., 1997; Odegaard et al., 1997; Asano et al., 2006). Wild-type *Agrobacterium rhizogenes* strains are routinely used to induce hairy root formation. However, such a method may make genetic manipulation of hairy roots difficult because of the large size of the root-inducing (Ri) plasmid in *A. rhizogenes*. Here, we describe an efficient and simple hairy root induction system for Chinese cabbage, employing *Agrobacterium tumefaciens* that harbors a binary vector carrying the *rolABC* genes.

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MATERIALS AND METHODS

Plant Material

Seeds of a commercial Chinese cabbage cultivar, 'Seoul' (Kyoungshin Seed Co., South Korea), were sterilized and cultured as previously described (Zang et al., 2008). Ten-day-old seedlings were used for *Agrobacterium* infection.

Construction of Plant Expression Cassette

Plasmid pPZPROL (Hong et al., 2005) contains the *rolABC* loci from T_L-DNA of pRiA4 harbored in the agropine-type *Agrobacterium rhizogenes* strain A4. A 4.4-kb *Eco*R I fragment carrying the *rolABC* genes was sub-cloned into pC11, a derivative of the binary vector pCAMBIA1301 (CAMBIA, Canberra, Australia) that carries multiple cloning sites flanked by the CaMV35S promoter and the nopaline synthase (NOS) terminator, the plant selectable marker *hpt* gene, and the *gus* reporter gene within T-DNA. This construct was named pCROL (Fig. 1). Constructs pPZPROL and pCROL and vector pC11 were transformed into *A. tumefaciens* strain GV3101 by the freeze-thaw method (Holsters et al., 1978). Transformants that grew on selection LB plates containing spectinomycin (50 mg L^{-1}, for pPZPROL) or kanamycin (50 mg L^{-1}, for pC11 and pCROL) were analyzed with restriction enzymes for the presence of those plasmids. Confirmed clones were then used for plant transformation.

Infection with *Agrobacterium tumefaciens*

A. tumefaciens GV3101 (pPZPROL) and GV3101 (pCROL or pC11) were cultured to an OD_{600} value of approx. 1.0 in LB-spectinomycin and LB-kanamycin, respectively. The cells were harvested and re-suspended in MS medium supplemented with 100 μM acetosyringone (pH 5.2). Cells of GV3101 (pPZPROL) were adjusted to one of three concentrations (OD_{600} value of 1, 2, or 3). After the cells were kept

Abbreviations: DEPC, diethylpyrocarbonate; GUS, β-glucuronidase; MS medium, Murashige and Skoog medium; RT-PCR, reverse transcription polymerase chain reaction.

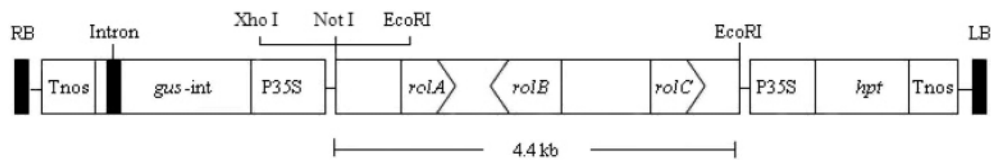

Figure 1. T-DNA region of expression vector construct, pCROL, in pC11 vector backbone. RB, right border; LB, left border; P35S, CaMV35S promoter; *gus*-int, β-glucuronidase gene containing intron; Tnos, nopaline synthase terminator; *rolA*, root-inducing locus A; *rolB*, root-inducing locus B; *rolC*, root-inducing locus C; *hpt*, hygromycin phosphotransferase.

at room temperature for 1 h, they were used to inoculate a stem tip (one wounding site) and two leaf veins (two to three wound sites each) on 10-day-old seedlings using a 10-mL syringe needle. Infected seedlings were grown under a 16-h photoperiod at 21°C.

Production and Maintenance of Hairy Roots

Roots (1 to 2 cm long) appeared at the infection sites after two to four weeks of incubation. They were transferred to a 1/2 MS medium (half-strength MS basal salts, 2% sucrose, and 0.8% agar; pH 5.8) supplemented with 300 mg L^{-1} cefotaxime and were kept in the dark. Root tips (3 to 5 cm long) were sub-cultured onto the same media type every two weeks. After two months of subculturing, the axenic hairy roots were transferred to a cefotaxime-free 1/2 MS medium and tested for liquid adaptability. For liquid culture, three 5-cm-long root tips were inoculated into 30 mL of a sterilized 1/2 MS medium in a 50-mL Erlenmeyer flask.

Molecular and Histochemical Characterization of Hairy Roots

Genomic DNA was isolated using CTAB method (Murray and Thompson, 1980). Total RNA was isolated using Trizol reagent (Sigma-Aldrich, Inc., MO, USA) according to the manufacturer's instructions. PCR and RT-PCR analyses were performed to verify the presence and expression of the transgene, using Ex Taq DNA polymerase (Takara, Japan) and One-Step RT-PCR Kit (ReddyMix version; ABgene, Surrey, UK), with the *rolB* gene primer set of 5′-ATG GAT CCC AAA TTG CTA TTC CTT CCA CGA-3′ (forward) and 5′-TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC-3′ (reverse). All the conditions were the same as before except for the annealing temperature at 62°C (Zang et al., 2008). Amplification products were analyzed by 1% agarose gel electrophoresis. The expected product size was about 789 bp (Fig. 2). Gus assay of the hairy roots essentially followed the method previously described (Jefferson RA, 1987).

RESULTS AND DISCUSSION

Induction of Hairy Roots

When control seedlings were infected with *Agrobacterium tumefaciens* GV3101 that harbored the empty vector construct pC11, a very few weak roots formed from the infection sites (Fig. 3A). However, hairy roots emerged two to four weeks after infection with either GV3101/pPZPROL or GV3101/pCROL (Fig. 3B). These were thicker and grew much faster than the roots formed from control seedlings. The frequency of hairy root induction was about 30% for all the constructs as compared with about 8% from the control infection (Table 1). Neither the *Agrobacterium* concentrations nor the binary vectors pPZPROL and pCROL made any significant difference in the frequency of

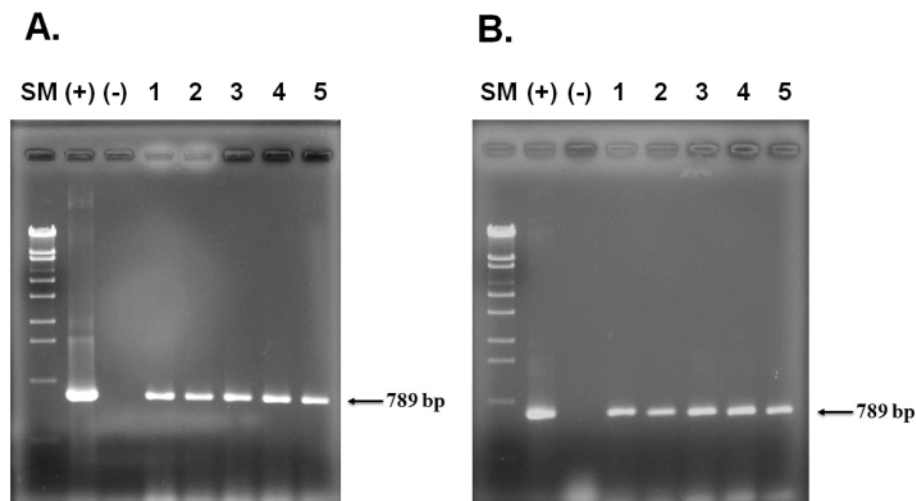

Figure 2. Analysis of transgenic hairy roots. **A,** PCR; **B,** RT-PCR. *rolB* primer was used. SM denotes molecular weight marker. Positive (+) and negative (−) controls indicate chimeric plasmid construct used for plant transformation and DNA or RNA purified from wild-type Chinese cabbage roots, respectively. Lines 1, 2, 3, 4, and 5 were randomly selected, with fast-growing hairy roots, as produced from GV3101/pPZPROL-infected seedlings.

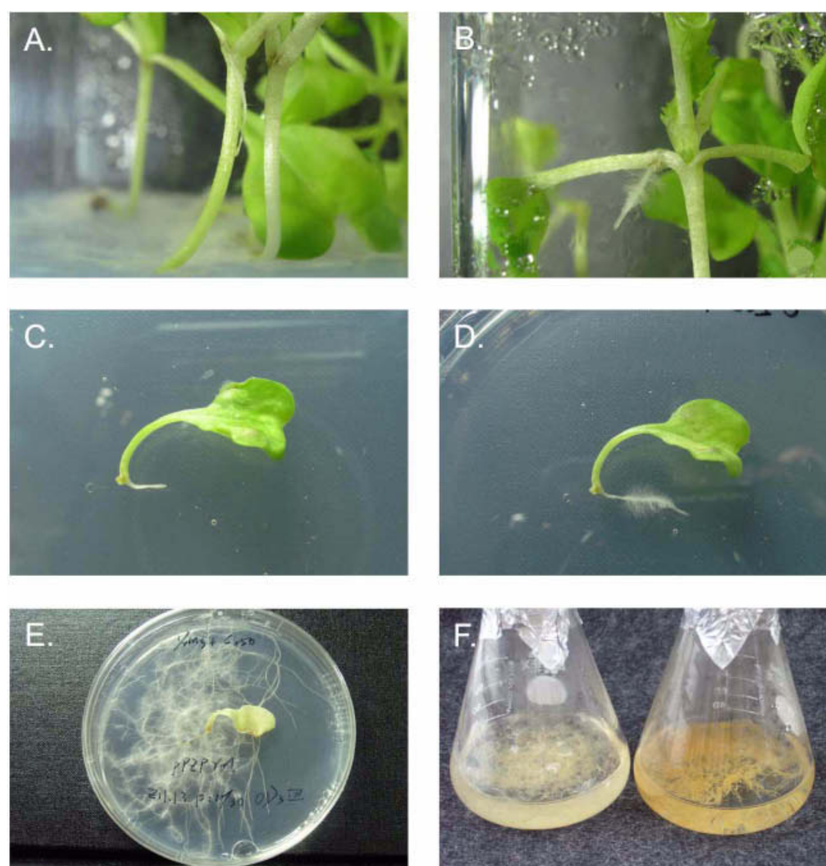

Figure 3. Representative procedure for hairy root production. **A,** non-hairy root from GV3101/pC11-infected seedlings; **B,** hairy root emerging at infection site on petiole of cotyledon 2 weeks after infection with GV3101/pPZPROL; **C,** hairy root together with cotyledon transferred to 1/2 MS medium containing 300 mg L^{-1} cefotaxime; **D,** hairy root producing root hairs 1 d after transfer to subculture medium. **E,** hairy root 15 d after transfer to subculture medium in Petri plate (9-cm diam.); **F,** fast-growing hairy root lines in liquid 1/2 MS medium, sub-cultured at 1-month intervals (left) or without subculture for 3 months (right).

Table 1. Hairy root formation from Chinese cabbage seedlings infected by *Agrobacterium tumefaciens* harboring different constructs.

Binary vector	No. of infected seedlings	No. of fast-growing hairy roots	No. of slow-growing hairy roots	Root formation efficiency (%)[c]
pPZPROL[a]	100	5.7±1.5	23.0±1.7	28.7±3.2
pPZPROL	100	5.3±2.1	26.7±3.8	32.0±5.1
pPZPROL[b]	100	5.0±1.1	26.3±5.5	31.1±6.4
pC11	100	0	8.3±5.5[d]	8.3±5.5
pCROL	100	4.3±1.5	23.3±1.2	27.7±2.1

Note: *Agrobacterium tumefaciens* strain is GV3101; [a] value for OD_{600} is 1; [b] value for OD_{600} is 3, value for OD_{600} of others is 2; [c] root formation efficiency is the total number of induced roots out of the total number of infected seedlings; [d] the number of adventitious roots produced. Data were collected 1 month after infection. Three replications were performed for each construct. Data represent means ± standard deviation.

hairy roots.

Maintenance of Hairy Roots

When the hairy roots were about 2 cm long, they were transferred to a 1/2 MS medium containing 300 mg L^{-1} cefotaxime. To establish axenic hairy roots, we sub-cultured 3- to 5-cm-long root tips every week, because those tissues were generally free of *Agrobacterium*. During this process, several lines were initially sub-cultured on one plate to save space. After one to two months, they were sub-cultured into a cefotaxime-free medium for another month. At this time, 10 fast-growing lines were transferred to a 1/2 MS liquid medium to test for the liquid adaptability. All of them grew very well in the liquid media (Fig. 3F). They were then sub-cultured after one or two months, although sub-culturing at a shorter interval would have been better. If the interval was longer than three months, the hairy roots died due to the lack of nutrients and air (Fig. 3F).

Growth Characteristics and Morphology of Hairy Root Lines

The *rolABC* hairy root lines exhibited a wild-type hairy root phenotype when induced by *A. rhizogenes* on either solid or liquid media. Those lines displayed dissimilar

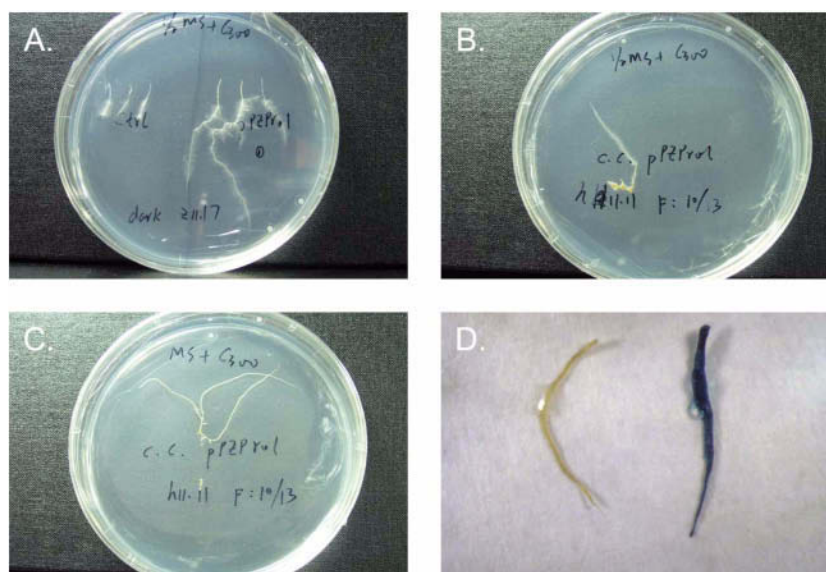

Figure 4. Hairy root phenotypes. **A,** comparison between non-hairy root (i.e., from normal tissue-cultured plant) and fast-growing hairy root; **B,** slow-growing hairy root with root hairs; **C,** slow-growing hairy root without root hairs; **D,** GUS assay for root from GV3101/pC11-infected seedlings (left) and for fast-growing hairy root from GV3101/pCROL-infected seedlings (right).

growth patterns, with some growing very fast (Fig. 4A). Fast-growing hairy roots usually appeared at the infection sites earlier than slow-growing hairy roots (Fig. 3B). After being separated from the plant and transferred to a 1/2 MS medium (Fig. 3C), these fast-growing hairy roots could elongate by more than 1 cm per day (Fig. 3D), with some occupying the Petri plate within a half month (Fig. 3E). They are highly branched with long, white root hairs. In contrast, other hairy roots grew slowly (Fig. 4B, C). They were usually not branched, and some did not have visible root hairs (Fig. 4C). All of the fast-growing and slow-growing hairy roots were weak, and they were easily broken when separated from the solid media.

Molecular and Histochemical Characterization of Hairy Roots

Because slow-growing hairy roots are not useful for experiments, we focused only on fast-growing hairy root lines, randomly selecting five independent lines that were transformed with GV3101/pPZPROL. PCR and RT-PCR analyses showed that all five lines contained and expressed the *rolB* transgene (Fig. 2 and 3). This demonstrated that *rolABC* expression was sufficient for hairy root induction as a visual selection marker. We also used pCROL to induce and screen hairy roots with additional markers HPT and GUS, together with pC11 as a negative control. Ten lines of fast-growing hairy roots were subject to GUS activity assay. More than 90% of them showed blue color. The GUS-negative lines may have been caused by GUS gene silencing. Next, we tested the GUS-positive lines for hygromycin resistance and found 100% survival. Roots that had formed from the seedling infected with GV3101/pC11 did not show any positive GUS activity or hygromycin resistance. The development of those non-hairy roots may result from wounding, which may induce their initiation and stimulate adventitious root production (LaRue, 1941).

CONCLUSION

Hairy root can be easily produced from young seedlings of Chinese cabbage by *Agrobacterium tumefaciens*-mediated transformation. The *rolABC* binary vectors that we used here may facilitate the hairy root-dependent metabolic engineering of secondary metabolites when that phenotype serves as a direct selection marker. Our fast-growing hairy roots had excellent liquid adaptability in the half-strength MS medium. We previously used a hairy root production system to metabolically engineered Chinese cabbage for indole glucosinolates (Zang, 2008). The protocol described here offers several advantages over earlier methods: 1) ease of gene manipulation and ectopic expression when a target gene is inserted into the binary vector, 2) increase in the number of transgenes by using a co-transformation vector, 3) time saved by selecting a fast-growing hairy root line, and 4) ease of establishing an axenic line by sub-culturing the hairy root tips at short intervals.

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