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An Efficient Plant Regeneration and Transformation System of *Robinia pseudoacacia* var. *umbraculifera* for Phytoremediation

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ABSTRACT *Robinia pseudoacacia* var. *umbraculifera*, commonly called umbrella black locust were regenerated after co-cultivation of internode segments with *Agrobacterium tumefaciens* which included yeast cadmium factor 1 (YCF 1) gene. The tolerance to cadmium and lead for plants can be increased by the YCF1 gene expression. Moreover, the recent studies have shown that YCF1 gene transgenic plants increase the accumulation of cadmium and lead into plant vacuoles. The effect of plant growth regulator such as 2,4-dichlorophenoxyacetic acid (2,4-D), *a*-naphthaleneacetic acid (NAA), 6-benzyladenine (BA), and thidiazuron (TDZ) were studied to evaluate the propagation of plants through internode explants. The efficient induction of multiple adventitious shoots and callus were observed on a medium supplemented with 0.1 mg/L TDZ + 0.2 mg/L BA. To induce shoot elongation and rooting, regenerated shoots were transferred into basal MS medium without any plant growth regulator. Successful *Agrobacterium tumefaciens* mediated transformation was obtained by 20 min vacuum-infiltration with 50 μM acetosyringone on the optimal multiple shoot induction medium with 30 mg/L hygromycin and 300 mg/L cefotaxime. To confirm the integration and expression of transgene, Polymerase Chain Reaction (PCR) and Reverse Transcriptase PCR (RT-PCR) were performed with specific primers. The frequency of transformation was approximately 18.94%. This study can be used to genetic engineering of phytoremediator.

Introduction

The selective improvement of individual traits of tree ussing genetic engineering techniques cut down expenses and time compared with conventional breeding system (Giri et al. 2004). Also, the desired traits of the parent lines are no loss during that process. *Agrobacterium tumefacience* mediated transformation has efficiently and successfully provided introducing foreign genes into plants. This method has been the preferred because of its simplicity and stable integration of wanted genes. A lot of plant species including a woody plant has been found susceptible to *Agrobacterium* infection (De Cleen and Deley 1976). The lack of efficient in vitro regeneration system, suitable gene vector and a selection system is a main problem to get transformed plants.

Heavy metal contaminants such as lead and cadmium have threatened the health of human. These heavy metals are very toxic to human even though at low concentration (Vido 2001). Moreover, heavy metal contaminant sites are widespread and easily found on abandoned mine and industrial waste nearby urban area. Therefore, these contaminant components have to remove from the environment to avoid environmental and human health problem. However, physical or chemical cleanup

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methods of contaminated sites are very expensive. Recently, phytoremediation has been proposed because of cost effective and environmentally friendly technology. Function of YCF1 (yeast cadmium factor 1) gene such as resistance to cadmium, arsenic, and stibium has been reported (Ghosh 1999). Also, lead and cadmium can be accumulated into plant vacuole by YCF1 expressing transgenic plants (Song et al. 2003).

Robinia pseudoacacia var. *umbraculifera* classified within Leguminosae, commonly called umbrella black locust is a nitrogen fixing leguminous tree. Moreover, this tree has agroforestry qualities such as outstanding ornamental effect. This tree has been also adapted well on environmental bad condition such as drought, high light intensity, and air pollutants, so can be used for environment restoration (Zaragoza et al. 2004). This tree has a fast growing rate, large biomass, and easy cultivation system. Also, there have no flowers. It can be advantage for safety of living genetically modified organism (LMO). In this regard, *R. pseudoacacia* var. *umbraculifera* is very powerful tool for phytoremediator.

In vitro regeneration system of *R. pseudoacacia* has been studied and reported well from Chalupa (1983), Davis and Keathely (1987), Merkle and Wiecko (1989), Arrillaga and Merkle (1993), Han et al. (1993), Hosseini-Nasr and Rashid (2003). *R. pseudoacacia* transformation system has been achieved by Igasaki et al. (2000), Zaragoza et al. (2004). Rarely, morphological abnormalities during plant transformation were reported (Han et al. 1993). However, there were no reports about regeneration and/or transformation of *R. pseudoacacia* var. *umbraculifera*. The overall goal of this study was to develop efficient regeneration system and YCF1 gene transformation protocols of *R. pseudoacacia* var. *umbraculifera* without morphological abnormalities, applicable for phytoremediation purposes.

Materials and Methods

Plant Materials

In June, young internodes of *R. pseudoacacia* var. *umbraculifera* were collected from several year-old plants grown at the Cheonan Agricultural High School. Internodes were surface sterilized by soaking in 75% EtOH for 1 min and 20% sodium hypochloride containing 0.1% Tween 20 for 10 min, and then they were washed three times with sterile water. The explants were cut into pieces approximately 5 mm × 5 mm. Following sterilization treatments, explants were placed in 9 cm plastic petridishes containing 25 ml of Murashige and Skoog medium (MS) (Murashige and Skoog 1962) supplemented with various combinations of plant growth regulator, such as 2.4-D (2,4-dichlorophenoxyacetic acid), BA (6-benzyladenine), TDZ (thidiazuron), and NAA (α-naphthaleneacetic acid) with 100 mg/L myo-inositol, 1 mg/L thiamine HCL, 0.5 mg/L pyridoxine HCL, 0.5 mg/L nicotinic acid, 2 mg/L glycine, 30g/L sucrose, and 8g/L agar. The pH was adjusted to pH 5.8 prior to autoclaving at 121℃ for 20 min.

Callus Induction and Regeneration of Multiple Shoots

To determine the optimal concentration of plant growth regulator for callus and multiple adventitious shoot induction from internode explants, 25 internode segments with three replications were placed on MS medium containing 2,4-D (0, 0.1, 0.2, 0.5 1 mg/L), BA (0, 0.1, 0.2, 0.5 1 mg/L), TDZ (0, 0.1, 0.2, 0.5 1 mg/L), NAA (0, 0.1, 0.2, 0.5 1 mg/L), 2,4-D + BA or BA + TDZ combination supplemented with 30 g/L sucrose and 8 g/L agar. Explants were cultured for 4 weeks under white fluorescent lights with 16-h photoperiod at 25℃ in a growth room. After 4 weeks culture, callus induction and regeneration rates were recorded by counting the number of callus and shoots per explants, and optimal plant growth regulator condition for regeneration was used for plant transformation. The tissues were subcultured every 4 week. After 8 weeks culture from initiation, induced shoots were transferred to basal MS medium for shoot elongation and root induction. Plantlets were transferred into plug trays in a potting mixture of peat moss, perlite and vermiculite (3:1:1 v/v) and kept under a 16-h photoperiod at 25℃ in a growth room. Two months after outplanting, plants were transferred to the greenhouse.

Transformation

Agrobacterium tumefaciens strains LBA4404 containing pCambia vector were used in preliminary transformation system.

For enhanced the expression of YCF 1, four copies of CaMV 35s promoter were inserted. *Agrobacterium tumefaciens* were grown at 28℃ in YEP medium in the presence of 50 mg/L kanamycin and 50 mg/L rifampicin. Agrobacterium cells were harvested, and resuspended using liquid basal MS medium at an OD_{600} of 0.6. To determine optimal vacuum-infiltration time, 1600 internode segments of *R. pseudoacacia* var. *umbraculifera* were vacuum-infiltrated for 10, 20, 30, 60, and 120 min with 50 μM acetosyringone. Tissues were then blotted with sterile filter paper, and co-cultivated on optimal multiple shoot induction medium with *Agrobacterium* without any antibiotics for 48hr in continuous dark condition. After then, tissues were transferred and cultured to optimal multiple shoot induction medium with 30 mg/L hygromycin and 300 mg/L cefotaxime in growth room. Tissues were subcultured every 4 weeks. Induced shoots were excised from explants, and then transferred to basal MS medium with 30 mg/L hygromycin for elongation and rooting.

Molecular Analysis

Genomic DNA of transgenic *R. pseudoacacia* var. *umbraculifera* was isolated from nontransformed plants and putative transformed plants using CTAB extraction protocol (Chaudhary et al. 1999). PCR was performed in an Eppendorf Mastercycler personal PCR system. PCR was conducted using 5'-ACTACCGTAAA GCTCGAGAAAATGGCTGGTAAT-3' and 5'-CTTGCCTAAG TGACGTGACGTCTCCTT-3' as forward and backward primers chosen from the YCF1 sequence. The amplification conditions were maintained at 94℃ for 5 min; 28 cycles of 94℃ for 30 sec, 62℃ for 30 sec, 72℃ for 2 min, and a final extension of 5 min at 72℃.

To confirm expression of introduced YCF1 gene, RNA was extracted from wild type (non-transgenic plants) and putative transgenic plants using TRIZOL, and cDNA was synthesized from a mRNA template using reverse transcriptase. RT-PCR was performed with specific primers taken from YCF1 gene; 5'-CATGAGTGCGTTCTATCCCTCTAT-3' and 5'-CCACCTT CGGTTAGTTGGGCATCT-3'. As a control for the RT-PCR, ssu (small subunit of RUBISCO) was used with followed primer sequences: forward-'5-CGGCTTGGAGGCGATAAAACTGAT-3', reverse-'5-CGCGGCCGAGGTACTATGATGGAC-3'. Amplification was carried out with the following procedures: an initial denaturation of 4 min at 94℃, 28 thermal cycles consisting of denaturation (94℃ for 30 sec), annealing (56℃ for 30 sec), and extension (72℃ for 1.5 min). Final extension was performed at 72℃ for 5 min. PCR products were visualized by ethidium bromide staining after electrophoresis in a 1% agarose gel, and photographed under UV light.

Results and Discussion

Callus and Multiple Shoot Induction

Adventitious shoots can be induced through directly or indirectly from explants. Indirect shoot organogenesis from explants occurred from callus or somatic embryo. In this study, formation rates of callus and multiple adventitious shoot were compared in MS medium supplemented with 2,4-D, BA, TDZ, NAA, 2,4-D + BA or BA + TDZ combination (Table 1). Generally, 2,4-D, TDZ, and NAA was more effective for callus induction than BA only used. Especially, callus was induced well 0.5 and 1 mg/L 2,4-D only, or BA + TDZ combination condition. Cultures initiated without any plant growth regulator showed only 3% callus production. The best callus induction (99%) response occurred with cultures on medium supplemented with 0.1 mg/L TDZ + 0.2 mg/L BA from internode segments.

Because of cytokinin-like activity in woody plant tissue culture, TDZ can promote cell division and differentiation (Huetteman & Preece, 1993). TDZ also has been reported to be effective to induce meristematic centres in the treated tissue (Beattie and Garrett 1995; Hutchinson et al. 1996). In case of multiple adventitious shoot regeneration, the best response (48%) showed in 0.2 mg/L BA + 0.1 mg/L TDZ combination. When only 2,4-D, BA, NAA or TDZ was used for shoot induction, only 0, 2-11, 0-4, or 6-24% were exhibited, respectively. When TDZ concentration was over 0.5 mg/L, shoot induction rate was dramatically decreased. Explants exhibited swelling within 1 week of explanting in optimal shoot induction medium. Multiple shoot formation was visible after 3-4 weeks in

Table 1. Effects of plant growth regulator (BA 2,4-D, TDZ, NAA, BA+2,4-D, BA+TDZ) on callus production and multiple shoot induction derived from internode segments of *R. pseudoacacia* var. *umbraculifera*

Treatment (mg/L)		Percentage of callus formation					Percentage of multiple shoot induction				
		BA 0	0.1	0.2	0.5	1	BA 0	0.1	0.2	0.5	1
2,4-D	0	3	39	50	38	29	0	6	11	5	2
	0.1	20	19	34	38	51	0	8	11	6	4
	0.2	54	45	37	41	35	0	7	7	4	3
	0.5	80	70	45	54	25	0	6	8	4	2
	1	88	71	40	59	64	0	3	5	2	1
TDZ	0.1	87	93	99	91	88	24	36	48	31	15
	0.2	89	93	95	89	79	14	28	32	26	16
	0.5	89	91	93	87	76	10	13	18	11	8
	1	79	83	89	76	71	6	7	11	5	3
NAA	0.1	69	-	-	-	-	0	-	-	-	-
	0.2	74	-	-	-	-	0	-	-	-	-
	0.5	72	-	-	-	-	4	-	-	-	-
	1	76	-	-	-	-	0	-	-	-	-

optimal shoot induction medium (Figure 1A). As a result, callus and multiple shoot formation were performed in the same medium condition (Figure 1B).

To promote shoot elongation and rooting in the current study, formed shoots (1-1.5 cm) were excised and transferred to basal MS medium without any plant growth regulator because TDZ reported inhibits shoot elongation in some woody plant species (Huetteman and Preece 1993). For example, efficient regeneration shoots using cranberry leaf explants were showed on media with TDZ + 2iP, but adventitious shoots were transferred onto a basal medium without any plant growth regulator for shoot elongation (Qu 2000). In Lingonberry leaf explants, adventitious shoot induced with TDZ exhibited inhibition of shoot elongation caused by TDZ. To overcome this problem, shoot cultures were transferred to a shoot elongation medium containing zeatin (Debnath 2003; Debnath 2005). Similarly, Chitra and Padmaja (2005) emphasized the importance of two step procedure to promote shoot formation of mulberry on TDZ based medium, and then formed shoots were transferred to a secondary medium with BAP to elongate shoots.

Elongated shoots were rooted well on basal MS medium (Figure 1c). Even though IAA (Indole-3 acetic acid) was used to promote root, there was no significantly different with no plant growth regulator treatment (data not shown). Shoot elongation and rooting occurred simultaneously on basal MS medium

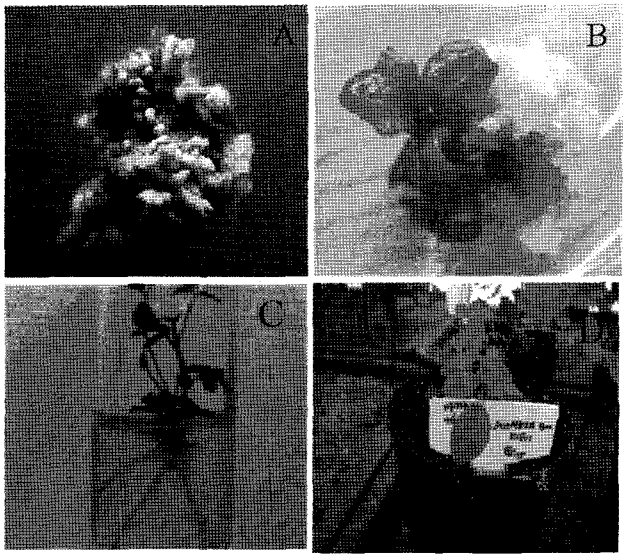

Figure 1. In vitro callus and multiple shoot induction from *R. pseudoacacia* var. *umbraculifera* (A) Induction of multiple adventitious shoots on MS + 0.2 mg/L BA + 0.1 mg/L TDZ (after 4 wk). (B) Induction of callus and shoot from internode segments on MS + 0.2 mg/L BA + 0.1 mg/L TDZ (after 8 wk). (C) In vitro shoot elongation and rooting. (D) An acclimatized plant.

Transformation

1600 internode segments of *R. pseudoacacia* var. *umbraculifera* inoculated with *Agrobacterium* were vacuum infiltrated for 10, 20, 30, 60, and 120 min to develop optimal vacuum infiltration time. The most effective time (18.94 %) was 20 min (Figure 2). After 20 min, transformation rates were dramatically decreased. For selection and regeneration of transformed explants, internode segments were co-cultivated with *Agrobacterium* for 48 h and transferred onto optimal shoot induction media (0.1 mg/L TDZ + 0.2 mg/L BA) supplemented with 30 mg/L hygromycin and 300 mg/L cefotaxime. After 8 weeks of selection, the hygromycin resistant shoots were transferred to basal MS medium containing 30 mg/L hygromycin with no plant growth regulator to promote shoot elongation and rooting. After an acclimation in growth room, plantlets were transferred to soil (3 Peatmoss : 1 Perlite : 1 Vermiculite v/v) (Figure 1d).

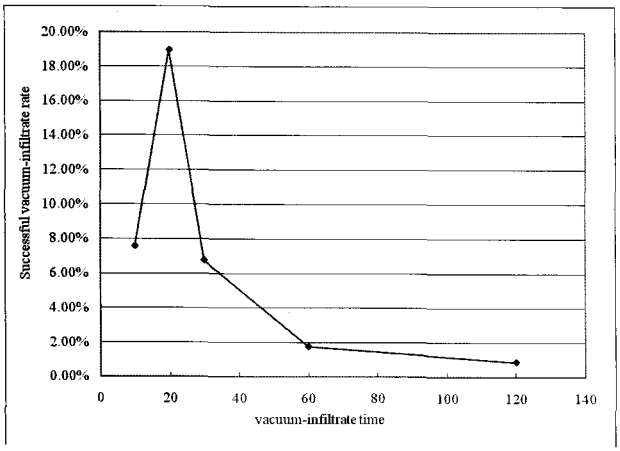

Figure 2. Evaluation of optimal vacuum-infiltration time for transformation of *R. pseudoacacia* var. *umbraculifera*. A total of 1600 internode segments were treated for vacuum-infiltration with various time as indicated. The numbers of hygromycin resistant shoots were recorded 4 weeks after induction of multiple adventitious shoots.

Molecular Analysis

To confirm the presence of the YCF 1 genes in putative transformants, PCR analysis was performed. A number of putative transformants were tested by PCR amplification of genomic DNA by using a set of specific primers. The expected size of the YCF 1 gene from 1, 4, 5, 6, 7, 8, 10, 11, 14, 15, and 16 independent lines was successfully detected, while there was no amplification in the non-transformed plants (Figure 3).

The expression level of the introduced gene was investigated by RT-PCR. For these analyses, RNA was isolated from 1, 4, 6, 7, 8, 10, 11, 14, 15, and 16 independent lines as well as a non-transformed plant. RT-PCR analysis showed an expected band in 7 transgenic plants, while expected band was not detected in the nontransformed plants (Figure 4). The mean transformation efficiency evaluated 18.94 %. Successful transformation of *R. pseudoacacia* var. *umbraculifera* by using the YCF1 gene was developed in this study. The protocol could be used for the genetic engineering phtoremediator.

(+): positive control
(-): negative control

Figure 3. PCR analysis for the presence of transgene in putative transformed plants. Genomic DNA was extracted from leaves of 16 putative transgenic lines and non-transformed plants. The negative control is DNA from non-transformed leaf tissue.

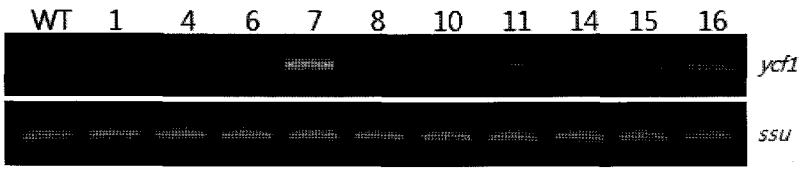

Figure 4. Expression of transgene in transgenic plants by RT-PCR analysis. RNA was isolated from non-transformed plants (wt), and 1, 4, 6, 7, 8, 10, 11, 14, 15, 16 lines observing expected band from PCR analysis.

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