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Transgenic Note

Genetic transformation of *Curcuma alismatifolia* Gagnep. using retarded shoots

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Abstract A protocol for regeneration and genetic transformation was established for *Curcuma alismatifolia* Gagnep. 'Chiang Mai Pink' using retarded shoots as explants. *In vitro* retarded shoots were cut into 0.5×0.5×0.5 cm blocks and co-cultivated with *Agrobacterium tumefaciens* strain AGLO harboring the binary vector, pBI121 or pBI121-*Ca-ACS1*. The explants were incubated in the bacteria suspension for 30 min. The explants and bacteria were cultured on MS medium for 2 days in darkness at 25°C for co-cultivation. Then, the explants were transferred onto MS medium containing 0.1 mg l^{-1} IAA, 4 mg l^{-1} IMA, 0.5 mg l^{-1} TDZ, 50 mg l^{-1} kanamycin and 500 mg l^{-1} vancomycin. The explants were subcultured every 2 weeks. After 4 weeks in culture, the explants with small shoot buds were transferred onto MS medium containing 50 mg l^{-1} kanamycin. Within 4 weeks, the shoots were separated and subcultured every 2 weeks on MS medium containing 0.1 mg l^{-1} IAA and 50 mg l^{-1} kanamycin. PCR analysis, histochemical GUS assay and Southern blotting of the regenerated plants confirmed transformation events. We obtained transformed plants within 3 months after co-cultivation with the bacteria and the transformation frequency exceeded 14%, which is suitable for practical use.

Key words: *Agrobacterium*, transformation, *Curcuma alismatifolia*, retarded shoots.

Curcuma, a genus of Zingiberaceae, is famous for its medicinal properties and as a spice plant as well as an ornamental plant in Thailand. *C. alismatifolia* Gagnep., which has wide variations in shape and bract color, has been cultivated for ornamental use. Cut flowers and tubers of *C. alismatifolia* are exported to many countries such as Japan, U.S.A, Netherlands and New Zealand (Pubuwpern 1992). It has become an important crop for breeding new varieties with novel or improved traits, due to its high economic value as a tropical ornamental (Prathepha 2000). Inflorescence of *C. alismatifolia* contains green basal bracts and coma bracts with pink color. Its vase life is 5 days determined by browning and wilting of the coma bracts. This browning may be related to ethylene production (Bunya-Atichart et al. 2004). One of the main objectives of research on *C. alismatifolia* is to shorten the breeding time for improving the flower quality such as inflorescence vase life. In this regard, genetic engineering could assist the genetic improvement of *C. alismatifolia* to create new varieties. Recently, genetic modification of plants using *Agrobacterium tumefaciens* has become a routine procedure for a large number of plant species (McCormick et al. 1986; Perl et al. 1996; Manoharan et al. 1998; Stafford 2000). *Agrobacterium*-mediated gene transfer has the advantage for allowing stable integration of defined DNA into the plant genome and generally results in a lower copy number, fewer rearrangements and more stable of expression over generations than free direct DNA delivery methods (Dai et al. 2001; Hu et al. 2003).

Curcuma is a monocotyledonous plant species. In recent years, significant advances have been achieved in *Agrobacterium*-mediated transformation for monocotyledonous species, especially cereal crops including rice, maize and wheat (Cheng et al. 2004). However, to introduce desirable traits by a gene transformation system, an efficient regeneration protocol is essential in *Curcuma*. The most important prerequisite for the method is the possibility to regenerate plants from callus tissues or explants, although comparative data, concerning tissue proposition and regeneration of *Curcuma* tissue, is very rare. Recently, Topoonyanont et al. (2004) reported success with micro propagation of *C. alismatifolia* from dwarf shoots, which were termed as "retarded shoots" by Shanks (1980) and Barrett (1982). Morphologically, leaf and stem expansion of the retarded shoots are reduced, but the stem and the shoots are maintained and can be seen with the naked eye. Thus

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retarded shoots are different from the meristemoid clusters, and organogenesis is obvious and only the expansion of the organ is reduced in retarded shoots. Since the meristem dome, an indicator of shoot bud, is at least visible, it is appropriate for transformation by targeting the cells of apical meristem. However, the genetic transformation of *Curcuma* has not been established yet. We report here the first evidence of *Agrobacterium*-mediated transformation of *C. alismatifolia* via organogenesis and how to optimize the high efficiency of the regeneration system. Moreover the antisense 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene isolated from *C. alismatifolia* was tried to introduce by this method, in an attempt to prolong vase life of the inflorescence after harvesting.

The retarded shoots of *C. alismatifolia* Gagnep. 'Chiang Mai Pink' was used as explants for genetic transformation by *Agrobacterium tumefaciens*. The retarded shoots were induced by the following procedure. Curcuma inflorescences grown in greenhouse were used as initial explants. After the lowest flower had bloomed, the inflorescences were cut and all bracts were removed, following the method of Topoonyanont et al. (2004). The coflorescences of each pouch were taken and placed in 250 ml glass bottles containing 25 ml MS medium (Murashige and Skoog 1962) containing 10 mg l^{-1} 6-bezylaminopurine (BA) and 0.1 mg l^{-1} 3-indoleacetic acid (IAA) supplemented with 3% sucrose and 7.5 g l^{-1} agar, adjusted to pH 5.8. The bottles were then placed in a culture room at 25±2°C with 14 hours photoperiod of 40 μmol m^{-2} s^{-1} provided by fluorescent light (Topoonyanont et al. 2004). After 1 month in culture, the tissues were subcultured in the regeneration medium (RS medium), which was MS medium containing 0.1 mg l^{-1} IAA, 0.5 mg l^{-1} thidiazuron (TDZ) and 4 mg l^{-1} imazalil (IMA) supplemented with 3% sucrose and 7.5 g l^{-1} agar, adjusted to pH 5.8. IMA is imidazole fungicide, which has been reported to have synergistic effect with different cytokinins (Werbrouck and Debergh 1995). In this research, Fungaflor® (Liro, Belgium) was used for IMA. The bottles were placed in a culture room at the same condition. Two to three months after subculture, the coflorescences were developed and reverted to retarded shoots. Retarded shoots were routinely subcultured in 25 ml RS medium.

Figure 1A shows construction of a plasmid vector for *C. alismatifolia* transformation. The plasmid pBI121-*Ca-ACS1* was reconstructed from the binary vector pBI121 (Clontech). A partial cDNA encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene from *C. alismatifolia* (Ca-ACS1) was inserted in antisense orientation at the unique *Bam*HI site between the cauliflower mosaic virus 35S promoter and β-glucuronidase gene (*GUS*) of pBI121. The *Agrobacterium tumefaciens* strain, AGLO (Lazo et al. 1991), harboring the binary plasmid pBI121-*Ca-ACS1* or pBI121 was used. The bacteria were cultured overnight in Luria-Bertani (LB) medium, containing 50 mg l^{-1} kanamycin and 50 mg l^{-1} rifampicin at 28°C. The density of the bacteria was adjusted to O.D. 600=0.5 with 10 mM glucose and 100 μM acetosyringone (AS). Retarded shoots of *C. alismatifolia* (Topoonyanont et al. 2004) were directly used as explants for transformation (Figure 1B-a). The explants were cut into 0.5×0.5×0.5 cm blocks and incubated in the bacteria suspension for 30 min and washed. Ten to fifteen explants treated with the bacteria were cultured horizontally in each petri dish (10 mm) on MS medium for 2 days in darkness at 25°C for co-cultivation (Figure 1B-b). Then they were transferred onto RS medium containing 50 mg l^{-1} kanamycin and 500 mg l^{-1} vancomycin (Sigma) and subcultured every 2 weeks. During the first month of culture, the explants continued to form leaves (about 3–4 small leaves). After 4 weeks in culture, the explants were transferred onto the selective elongation medium (SE medium), which is MS medium containing 50 mg l^{-1} kanamycin, supplemented with 3% sucrose and 7.5 g l^{-1} agar. Within 4 weeks, 1.5–2.0 cm of the elongated shoots were separated from the explants and transferred onto the rooting medium (IR medium), which is MS medium containing 0.1 mg l^{-1} IAA and 50 mg l^{-1} kanamycin, supplemented with 3% sucrose and 7.5 g l^{-1} agar and subcultured every 2 weeks. The explants and excised shoots were cultured in bottles containing RS, SE and IR media at 25±2°C with 14 hours photoperiod of 40 μmol m^{-2} s^{-1} provided by fluorescent light. Transformation frequency was calculated according to the following PCR and GUS assay.

Genomic DNA was isolated from fresh leaves according to the CTAB method of Doyle and Doyle (1990). Polymerase chain reaction (PCR) amplification was carried out to detect approximately 500 bp region of the *GUS* gene by using forward primer GUS1 (5′ CTG TAG AAA CCC CAA CCC GTG 3′) and reverse primer GUS2 (5′ CAT TAC GCT GCG ATG GAT CCC 3′). PCR reaction mixture contained 20 ng of the DNA, 200 μM dNTPs, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl at pH 9.0, 0.1% (v/v) Triton X-100, 0.25 μM of each primer and 2 units of Taq DNA polymerase (Promega) in a 20 μl final volume. Amplification was carried out with a thermal cycler (Perkin Elmer, Gene Amp PCR System 2400) for 30 cycles at 97 °C for 30 s, 62 °C for 30 s and 72 °C for 45 s. To avoid amplification of non-specific fragments, the reaction was carried out at a high annealing temperature (62°C). For Southern blot analysis, DNA (10 μg) was digested with *Hin*dIII, separated by electrophoresis in 0.8% agarose gel and transferred to a nylon membrane (Roche). The 2.0 kb *Bam*HI-*Sac*I fragment containing the *GUS* gene was purified from pBI121 using a QIAquick Gel Extraction

kit (QIAGEN), and was used as a probe (Figure 1A) for analysis. The DNA probe was labeled by a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche), according to the manufacturer's instructions. Hybridization and detection procedures were performed as described by the manufacturers. Hybridization was carried out at 68°C in a hybridization buffer. Consequently, the blot was washed twice at room temperature with 2×SSC and 0.1% SDS, and washed twice with 0.1×SSC and 0.1% SDS at 68°C. Detection was carried out by chemiluminescence with CDP-Star (Roche) and the blots were exposed to X-ray film (Amersham).

A histochemical GUS assay was conducted as described by Jefferson et al. (1987). Tissues were immersed in an X-Gluc solution, consisting of 2 mM XGluc, 100 mM Tris–HCl pH 7.0, 50 mM NaCl, 2 mM potassium ferricyanide and 0.1% (v/v) Triton X-100. Tissues were stained overnight in the dark at 37°C for 24 h, followed by washing through an ethanol series to remove chlorophyll. Assayed tissues were observed under a microscope and photographed.

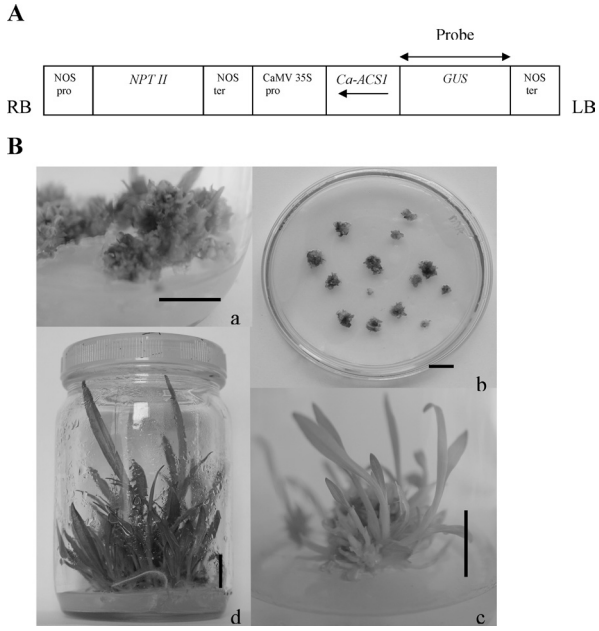

Figure 1. Transformation of *C. alismatifolia*. (A) Schematic representation between two T-DNA regions of pBI121-*Ca-ACS1*. RB, right border of the T-DNA; LB, left border of the T-DNA; NOSpro, nopaline synthase gene promoter; NOSter, nopaline synthase gene terminator; CaMV 35S, 35S promoter from cauliflower mosaic virus; *NPT II*, neomycin phosphotransferase gene; *Ca-ACS1*, antisense cDNA encoding ACC synthase gene from *C. alismatifolia*; *GUS*, β-glucuronidase gene. B, *Bam*HI; H, *Hin*d III; S, *Sma*I; X, *Xba*I. (B) Shoot regeneration from the retarded shoots of *C. alismatifolia* transformed by *Agrobacterium tumefaciens*. Retarded shoot clusters (a); Retarded shoots 1 week after transformation (b); Elongated shoots 4 weeks after transformation (c); Elongated shoots 8 weeks after transformation (d). Bar=1.0 cm.

The transformation frequency was evaluated by histochemical GUS activity and PCR analysis (Table1). Eight weeks after co-cultivation, 10 and 15% explants of 60 and 54 leaves from shoots transformed by pBI121 and pBI121-*Ca-ACS1* respectively, showed stable GUS expression. Elimination of *Agrobacterium* was checked on MS medium without antibiotics for 1 week and bacterial contamination was not detected. After about four weeks of culture on IR medium, prominent GUS activity was found in elongated leaves, stems and roots of the plants transformed by pBI121 and pBI121-Ca-ACS1 (Figure 2C).

Eight weeks after co-cultivation, PCR analysis was performed to confirm the presence of the *GUS* gene in regenerated shoots. The same shoot tested for GUS expression was used as PCR material. The PCR reaction revealed the presence of the GUS fragment with expected size of 500 bp in genomic DNA of each putative transgenic plant (Figure 2A). Transformation frequency by PCR was 15% in both regenerated plants of pBI121 and pBI121-*Ca-ACS1*. Four percent of pBI121 transformants amplifying PCR products did not express GUS activity. No amplified products were detected in non-transformed control plants.

Transgenic plants with pBI121-*Ca-ACS1*, expressing high GUS activity, were analyzed for Southern blotting by using the *GUS* gene as a probe. Genomic DNA, digested with *Hin*dIII, which was cut only one site within the introduced construct, produced multiple fragments in transformed plants, indicating that the foreign gene had been integrated into the genome of the putative transformed plants at multiple insertion sites (Figure 2B). In addition, different band patterns observed in 4 transgenic plants were derived from 4 independent transformation events.

Retarded shoots are a very important tissue for transformation of *Curcuma* due to their high ability to regenerate the whole green part of the plant body, including the flowers. This organ is a very tiny structure with leaf primordia and often confused with the complete shoot apex, containing the leaf primordial

Table 1. Transformation efficiency of *C. alismatifolia*, based on the number of retarded shoots as explants.

Period after infection	Number of explants showing GUS spots		Number of transformed shoots showing positive PCR	
	pBI121	pBI121-*Ca-ACS1*	pBI121	pBI121-*Ca-ACS1*
8 weeks(elongation)[a]	6/60 (10%)	8/54 (15%)	9/60 (15%)	8/54 (15%)

[a] PCR and GUS analyses on 60 and 54 shoots transformed by pBI121 and pBI121-*Ca-ACS1*, respectively

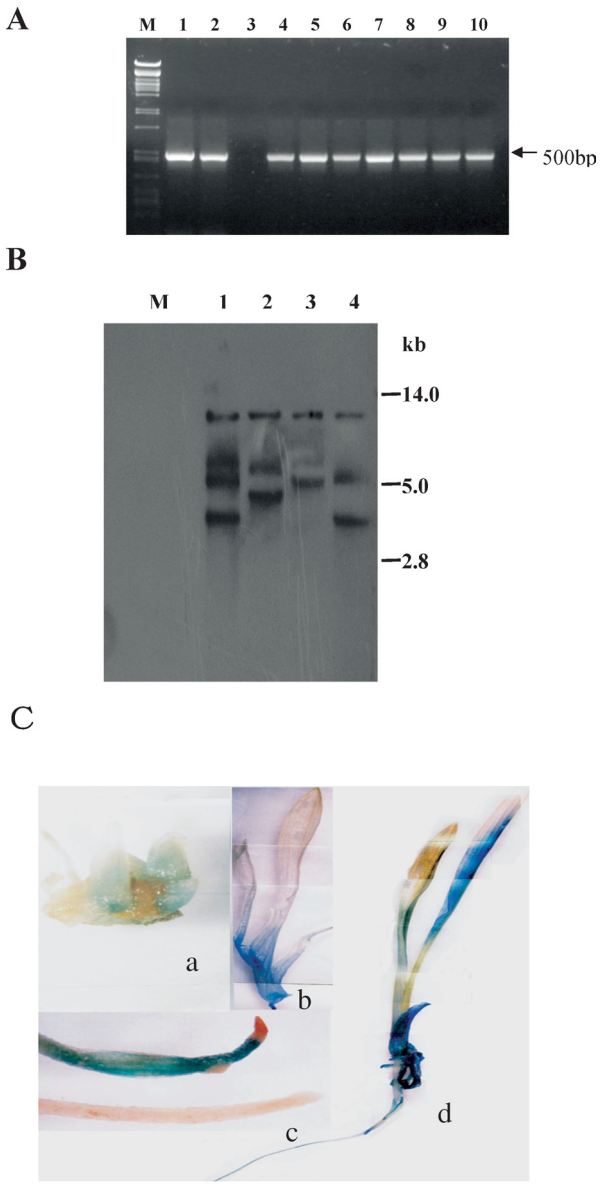

Figure 2. *GUS* gene expression in transgenic *C. alismatifolia*. (A) PCR amplification. Lane M, λ/*Pst*I molecular size marker; 1, plasmid pBI121; 2, plasmid pBI121-*Ca-ACS1*; 3, non-transformed plant; 4–10, transformed lines with pBI121-*Ca-ACS1*. Arrow indicates the PCR products of expected size after GUS gene amplified (500 bp). (B) Southern blot analysis. *Hin*dIII-digested genomic DNAs were used for templates and the *GUS* gene as a probe. Lane M, λ/*Pst*I molecular size marker; 1, non-transformed plant; 2 to 5, GUS-positive lines transformed with pBI121-*Ca-ACS1*. DNA sizes are indicated in kb. (C) Histochemical observation. Retarded shoots 2 weeks after transformation (a); Elongated shoot 4 weeks after transformation (b); Emerged root of transformed plant (upper) and untransformed one (below) after 4 weeks culture in IR medium (c); Whole plant transformed after 4 weeks culture in IR medium (d).

(Topoonyanont et al. 2004). In our transformation experiments, when transformation efficiency was calculated, based on the number of transgenic plants recovered by the number of the original intact retarded shoots, the transformation frequency with pBI121 and pBI121-*Ca-ACS1* was approximately 14%. This high frequency indicates that our system was an efficient method for selecting transgenic plants of *C. alismatifolia*. In addition, the procedure described here allowed to obtain a large number of transgenic *C. alismatifolia* plants from retarded shoots within 3 months. The transformation efficiency was comparable with other *Agrobacterium*-mediated transformation results for monocotyledonous species, such as rice (Aldemita and Hodges 1996), maize (Ishida et al. 1996) and wheat (Hu et al. 2003). This is the first report on *Agrobacterium tumefaciens*-mediated transformation in *C. alismatifolia*. An application of this established technique in *C. alismatifolia* might permit introduction of foreign genes for conferring quality improvement such as extension of vase life. Further investigation on the physiological and phenotypic changes of transgenic plants with *Ca-ACS1* gene is now under way in greenhouse.

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References

Aldemita RR, Hodges TK (1996) *Agrobacterium tumefaciens* mediated transformation of japonica and indica rice varieties. *Planta* 199: 612–617

Barrett JE (1982) *Chrysanthemum* height control by ancymidol, PPP 333 and EL-500 dependent on medium composition. *Hort Science* 17: 896–897

Bunya-Atichart K, Ketsa S, Doorn van WG (2004) Postharvest physiology of *Curcuma alismatifolia* flowers. *Postharvest Biol and Technol* 34: 219–226

Cheng M, Lowe BA, Spencer TM, Ye XD, Armstrong CL (2004) Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. *In Vitro Cell Dev Biol Plant* 40: 31–45

Dai S, Zheng P, Marmey P, Zhang S, Tian W, Chen S, Beachy RN, Fauquet C (2001) Comparative analysis of transgenic rice plants obtained by *Agrobacterium* mediated transformation and particle bombardment. *Mol Breed* 7: 25–33

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. *Focus* 12: 13–15

Hu T, Metz S, Chay C, Zhou HP, Biest N, Chen G, Cheng M, Feng X, Radionenko M, Lu F, Fry J (2003) *Agrobacterium* mediated large-scale transformation of wheat (*Triticum aestivum* L.) using glyphosate selection. *Plant Cell Rep* 21: 1010–1019

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nature Biotech* 14: 745–750

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in

higher plants. *EMBO J* 6: 3901–3907

Lazo GR, Stein PA, Ludwig RA (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Bio technol* 9: 963–967

Manoharan M, Sree Vidya CS, Lakshmi Sita G (1998) *Agrobacterium tumefaciens* mediated genetic transformation of hot chilli (*Capsicum annuum* L. cv.. Pusa jwala). *Plant Sci* 131: 77–83

McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch R, Fraley R (1986) Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. *Plant Cell Rep* 5: 81–84

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. *Plant Physiol* 15: 473–497

Perl A, Lolan O, Abu-Abied M, Holland D (1996) Establishment of an *Agrobacterium*-mediated transformation system for grape (*Vitis vinifera* L.): the role of antioxidants during grape-*Agrobacterium* interactions. *Nature Biotechnol* 14: 624–628

Prathepha P (2000) Screening of random primer to evaluate DNA diversity. *Songklanakarin J Sci Technol* 22: 8–13

Pubuwpern J (1992) The growth and development of *Curcuma*. M Sc Thesis, Chiang Mai University, p 82

Rademacher W (1991) Biochemical effects of plant growth retardants. In: Gausman HW (ed) *Plant Biochemical Regulators*. Marcel Dekker, NY, pp 169–199

Stafford HA (2000) Crown gall disease and *Agrobacterium tumefaciens*: a study of the history, present knowledge, missing information, and impact on molecular genetics. *Botanical Rev* 66: 99–118

Shanks JB (1980) Chemical dwarfing of several ornamental greenhouse crops with PPP 333. *Proc 7th Meeting Plant Growth Regulators Working Group*. Plant Growth Reg Soc Am June 1980. pp 46–52

Topoonyanont N, Chongsang S, Chujan S, Somsueb S, Nuamjareen P (2004) Micropropagation Scheme of *Curcuma alismatifolia* Gragnep. *Acta Hort* (inpress)

Werbrouck SPO, Debergh PC (1995) Imazalil enhances the shoot inducing effect of benzyladenine in *Spathiphyllum floribumdum* Schott. *J Plant Growth Reg* 14: 105–107