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-ACSI. 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 the method is the possibility to regenerate plants from tissue proposition and regeneration of callus tissues or explants, although comparative data, concerning tissue proposition and regeneration of *Curcuma* tissue, is very rare. Recently, Topoonyantont 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...
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 meristemoid, 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 Curcuma 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\(^{-1}\) 2,4-dichlorophenoxy-acetic acid (2,4-D) and 0.1 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. 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, Fungaflo® (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-ACS\(^{1}\) was reconstructed from the binary vector pBI121 (Clontech). A partial cDNA encoding 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene from C. alismatifolia (Ca-ACS\(^{1}\)) was inserted in antisense orientation at the unique BamHI 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-ACS\(^{1}\) 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 acetylsyringone (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 GUS\(^{1}\) (5’ CTG TAG AAA CCC CAA CCC GTG 3’) and reverse primer GUS\(^{2}\) (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 HindIII, separated by electrophoresis in 0.8% agarose gel and transferred to a nylon membrane (Roche). The 2.0 kb BamHI-SacI 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.

The transformation frequency was evaluated by histochemical GUS activity and PCR analysis (Table 1). 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 HindIII, 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
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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