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Genetic transformation in *Curcuma alismatifolia* Gagnep., *Petunia axillaris* and *Nicotiana tabacum* via *Agrobacterium tumefaciens*

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ABSTRACT

Gus gene was transformed into Siam tulip (*Curcuma alismatifolia* Gagnep.), petunia (*Petunia axillaries*) and tobacco (*Nicotiana tabacum*) using 2 types of plasmid (pSCV1.6 and pBI121) in *Agrobacterium tumefaciens*. Two days after co-cultivation with bacteria, *C. alismatifolia* Gagnep. were grown on a selective medium with 50 mg/l kanamycin (RM1). The *GUS* gene were detected by GUS histochemical assay and PCR analysis. The tobacco shoots and petunia shoots were grown on another selective medium with 50 mg/l kanamycin (RM2) and then their *GUS* genes were detected by GUS histochemical assay. In case of *C. alismatifolia* Gagnep., the blue spots indicating the presence of *GUS* gene were found at 0.83%. The gene transformation was also confirmed by the presence of DNA strip of CaMV 35S promoter primer using PCR analysis. The explants of *P. axillaris* and *N. tabacum* which transformed with pSCV1.6 showed GUS positive at 23.33% and 14.4%, respectively. The explants of *P. axillaris* and *N. tabacum* which transformed with pBI121 showed GUS positive present 13.33% and 3%, respectively. For tobacco and petunia, although blue spots could be found on all leaf surfaces transformed with plasmid pSCV1.6 and pBI121 but blue spots which found on plasmid pSCV1.6 gave a better indicate for detecting *GUS* gene.

Keywords: *Agrobacterium tumefaciens*, plant transformation, *GUS* gene, *C. alismatifolia* Gagnep.

INTRODUCTION

Curcuma is a genus of Zingiberaceae, a decorative flower which has economic importance in Thailand. It was compose of variable shapes and bract colours of each spicies. Curcuma has been exported to many countries such as Japan, U.S.A, Netherlands and New Zealand (Pubuwpern, 1992). At present, due to its high economic value as a tropical greenhouse ornamental, it has become an important target for breeding new varieties with novel or improved characteristics. Inflorescence of *C. alismatifolia* Gagnep. contains green basal bracts and several coma bracts.

The main objective of our work was to shorten the time taken for the improvement of quality. In this regard genetic engineering could assist the genetic improvement of *C. alismatifolia* Gagnep. as it enables specific traits to be added to highly selected genotypes. Recently, genetic modification of plants using *Agrobacterium tumefaciens* is a routine procedure for a large number of plant species (McCormick *et al.*, 1986; Perl *et al.*, 1996; Manoharan *et al.*, 1998; Stafford, 2000). In recent years, significant advances have been achieved in *Agrobacterium* - mediated transformation in monocotyledonous species especially cereal crops including rice, maize and wheat (Cheng *et al.*, 2004).

Three breeding methods are available in plants : 1) cross breeding 2) mutation breeding and 3) genetic modification. In cross breeding, new individuals originate from sexual reproduction, and hereditary elements from different plants are combined to create completely new gene combinations. Cross breeding cannot be used to add a new feature while leaving the recipient parent genotype otherwise intact, but mutation breeding can. The colour variants of elite genotypes are all obtained by mutation, either spontaneous or induced. Mutation breeding has limited potential, however, as it modifies existing pathways but it does not add new gene which is the power of genetic modification. We intend to use *Agrobacterium* - mediated genetic modification to add genes for developing quality of flower colour in the future.

Delivery of *Agrobacterium* T-DNA somatic cells and regeneration of transformed cells are essential for production of transgenic plants. Protocols for adventitious shoot formation (Robinson and Firoozabady, 1993) and somatic embryogenesis (Urban *et al.*, 1994) have been published. We decided to use *Agrobacterium*-mediated genetic modification because it is fast, genotype independent and avoid mutations among the regenerations.

For *P. axillaris* and *N. tabacum* which were studied about *gus* gene transformation because they were model plants. So, they were transformed *gus* gene compared to *C. alismatifolia* Gagnep

In this study, we reported the first evidence of *Agrobacterium*-mediated transformation in *C. alismatifolia* Gagnep. (monocotyledonous species) by using coinflorescense organ.

MATERIALS AND METHODS

Plant materials

The coinflorescences of Curcuma (*C. alismatifolia* Gagnep) cv. Chiang Mai Pink, the explants of tobacco and petunia were obtained from Plant Tissue Culture Laboratory, Department of Biology, Faculty of Science, Maejo University, Thailand. The coinflorescenses were sterilized by dipping in 70% ethanol for 30 seconds. And then soaking with 0.15% mercuric chloride solution for 10 minutes. After that washed three times with sterile distilled water. These sterilized explants were used for the transformation. Shoot regeneration medium (RM1) for the coinflorescences of *C. alismatifolia Gagnep* was MS medium (Murashige and Skoog, 1962) containing 10 mg/l benzyladenine (BA) and 0.1 mg/l indole-3-acetic acid (IAA) the regeneration medium (RM2) for leaf explant of tobacco and petunia was MS medium containing 0.1 mg/l IAA and 0.3 mg/l BAP .

Bacteria and vectors

Agrobacterium tumefaciens strain AGLO harboring the binary plasmids pSCV1.6 and pBI121 were used. The bacteria were cultured overnight in Luria-Bertani (LB) medium containing 100 mg/l kanamycin and 50 mg/l rifampicin, at 28°C.

Transformation

Agrobacterium tumefaciens strain carrying the designated plasmid were grown on LB containing 100 mg/l kanamycin and 50 mg/l rifampicin until reaching the optical density (at 600 nm) of 0.6-0.8. Then the bacterial solution was centrifuged at 6000 g for 5 minutes. The bacterial pellet was re-dissolved in MS-20 (MS medium with 2% sucrose supplemented with 100 µM acetosyringone and 0.1 M betain hydrochloride). Coinflorescences explants (0.5-0.8 cm in diameter) of *C. alismatifolia* Gagnep., leaf explants from tobacco and petunia (0.5-0.8 cm in diameter) were immersed in the bacterial solution at the O.D. adjusted concentration for 30 minutes while shaking at 80 rpm. Then, they were blotted dry on sterile filter paper. Coinflorescences explants of *C. alismatifolia* Gagnep. were co-cultivated with *Agrobacterium* on media (RM1) for 2 days while the leaf explants from tobacco and petunia were co-cultivated with *Agrobacterium* in media (RM2) for 2 days. Then, the explants were transferred to regeneration media, RM1 for *C. alismatifolia* Gagnep., RM2 for tobacco and petunia, containing both 50 mg/l kanamycin and 400 mg/l cefotaxim for selection of plasmids pSCV1.6 and pBI121. The shoots of *C. alismatifolia* Gagnep. were transferred to hormone free-MS containing the same antibiotic as described above.

Histochemical β-glucuronidase (GUS) assay

The leaves of *C. alismatifolia* Gagnep., tobacco and petunia were assayed for the expression of *gus* gene using the histochemical GUS assay. A histochemical GUS assay was conducted as described by Jefferson (1987). The leaves were immersed in an X-gluc solution, consisting of 2mM X-gluc, 100 mM Tris-HCl pH 7.0, 50 mM NaCl, 2 mM potassium ferricyanide and 0.1% (v/v) Triton X-100. All

tissue were stained in the dark at 37 °C for 24 h, followed by washing through an 70% ethanol to remove chlorophyll. Assayed tissue were observed under a microscope and photographed.

PCR analysis

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 detect approximately 194 bp region of the CaMV35S promoter by using forward primer CaMV35S1 (5'GCTCCTACAAATGCCATCA-3') and reverse primer CaMV35S2 (5'- GATAGTGGGATTGTGCGT CA-3'). PCR reaction mixture contained 20 ng of the DNA, 200 µM dNTPs, 25 mM $MgCl_2$, 20 mM Tris-HCl, 0.25 µM of each primer and 2 unit of Taq polymerase buffer in a 20 µl total volume. Amplification was carried out with a thermal cycler (Perkin Elmer, Gene Amp PCR 2400) for 30 cycles at 94°C for 1 minute, 67 °C for 1 minute and 72 °C for 1 minute. A final elongation step was carried out at 72°C for 5 minutes. PCR products were separated by electrophoresis on 1% agarose gels.

RESULT

Agrobacterium tumefaciens strain AGLO containing either plasmid pSCV1.6 or pBI121 were used to infect *C. alismatifolia* Gagnep., tobacco and petunia explants. After 8 weeks, the shoots of tobacco and petunia appeared from transformed explants while those of *C. alismatifolia* Gagnep.began to produce after 16 weeks. Adventitious shoots from each plant could produce roots in selective medium containing antibiotic while the control, non-transformed plants, could not produce root in this media. The leaves from all 3 transformed plants were used for GUS assay. In *C. alismatifolia* Gagnep., only transformed explants with pSCV1.6 could regenerate shoot. Only 13 regenerated shoot from pSCV1.6 transformed were PCR positive (Figure 1). Gus assay revealed that only 2 plantlets gave positive and transformation efficiency was 0.83% shown on Table 1 and Figure 2(A1).

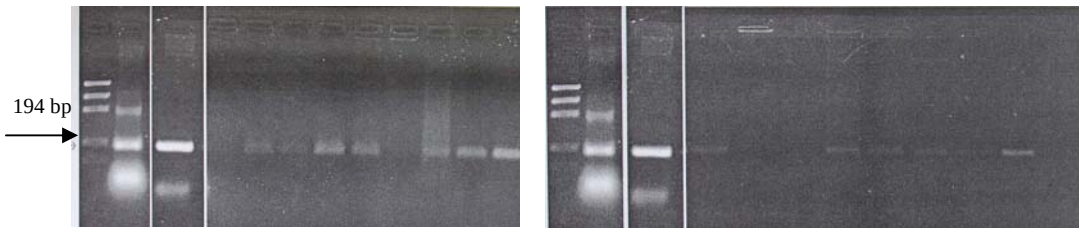

Figure 1 PCR reaction for CaMV35S promoter primer on coinflorescence in Curcuma by *Agrobacterium*-mediated transformation strain AGLO
M : 100 bp marker
PC1 : pBISCV1.6
PC2 : pBI121
NC : non transformation in Curcuma
1-19 : transformation in Curcuma by *A. tumefaciens* strain AGLO/Pscv1.6

Table 1 Transformation efficiency determined by GUS assay of *C. alismatifolia* Gagnep**.** after culturing in a selective media (RM1) for seven months.

Type of plasmids	Number of explants	Number of regenerated shoots	Number of shoot with PCR positive	Number of shoot with GUS positive	Transformation efficiency based on GUS assay (%)
AGLO/ pSCV1.6	240	19	13	2	0.83
AGLO/ pBI121	240	0	0	0	0

In tobacco, the explants transformed with pSCV 1.6 and pBI121 could grow and produce root in the selective media (RM2). The leaves from these transformed plantlets were assayed for GUS positive. It was shown that GUS positive in regenerated shoots were 14.4% and 3.3% for the transformed plasmids pSCV 1.6 and pBI121, respectively (Table 2 and Figure 2(B1) and 2(B2)).

Table 2 Transformation efficiency determined by GUS assay of tobacco after culturing in a selective media (RM2) for five months.

Type of plasmids	Number of explants	Number of regenerated shoots	Number of shoot with GUS positive	Transformation efficiency based on GUS assay (%)
AGLO/ pSCV1.6	90	20	13	14.4
AGLO/ pBI121	90	14	3	3.3

In case of petunia, the explants transformed with pSCV1.6 and pBI121 could regenerate shoot with transformation efficiency at 23.33 and 13.33%, respectively (Table 3 and Figure 2(C1) and 2(C2)).

Table 3 Transformation efficiency determined by GUS assay of petunia after culturing in a selective media (RM2) for five months.

Type of plasmids	Number of explants	Number of regenerated shoots	Number of shoot with GUS positive	Transformation efficiency based on GUS assay (%)
AGLO/ pSCV1.6	60	43	14	23.33
AGLO/ pBI121	60	41	8	13.33

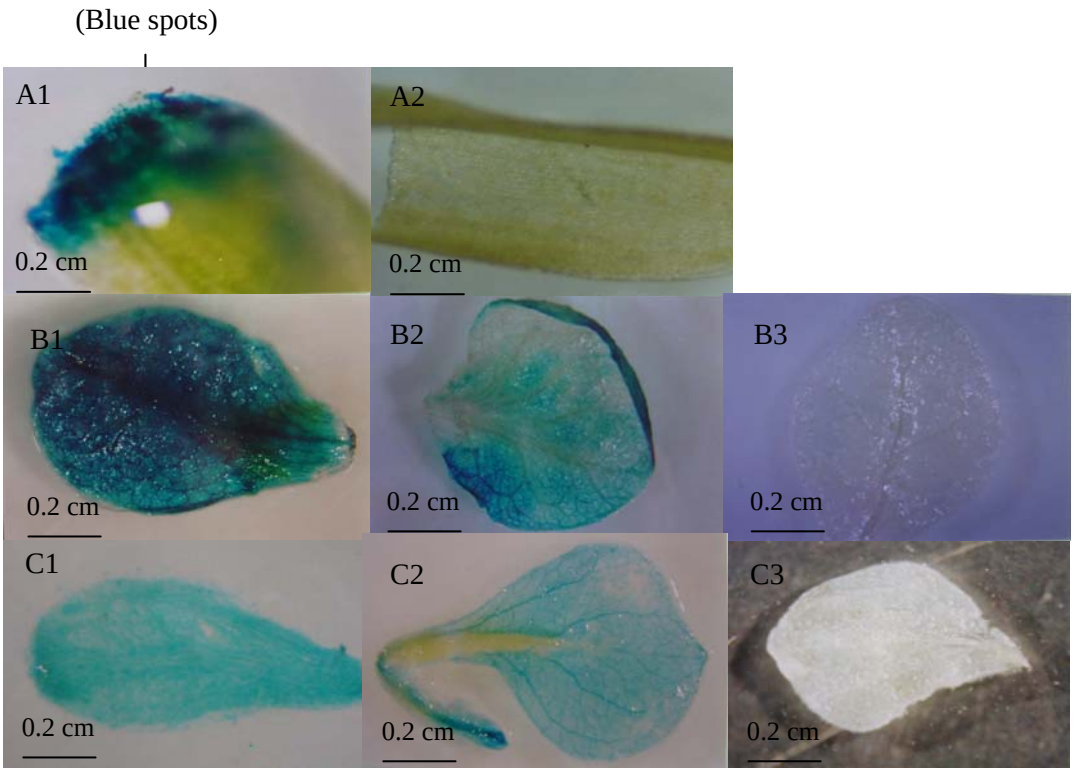

Figure 2 The Blue spots of histological observation of the *GUS* gene expression in transgenic plant with *Agrobacterium tumefaciens* strain AGLO

(A1) Blue spots on leaves of *C. alismatifolia* Gagnep. transformation with plasmid pSCV1.6
(A2) Nontransformation leaves of *C. alismatifolia* Gagnep.
(B1) Blue spots on leaves of tobacco transformation with plasmid pSCV1.6
(B2) Blue spots on leaves of tobacco transformation with plasmid pBI121
(B3) Nontransformation leaves of tobacco
(C1) Blue spots on leaves of petunia transformation with plasmid pSCV1.6
(C2) Blue spots on leaves of petunia transformation with plasmid pBI121
(C3) Nontransformation leaves of petunia

DISCUSSION

In *C. alismatifolia* Gagnep., the transformed explants with pSCV 1.6 could produce 19 shoots which grew on a selective media containing 50 mg/l kanamycin. However, only thirteen clones gave PCR positive with specific primer to CaMV35S. The GUS positive clones showed integration of the transgene in the *C. alismatifolia* Gagnep. genotype and phenotype (Figure 2(A1)). In case of tobacco and petunia, they were transformed by two types of plasmid. The gene transformation in their shoots was confirmed by gus histochemical assay and the presence of blue spots on all leaf surface. The blue spots were highly significant difference between transformed by plasmid pSCV1.6 and plasmid pBI121. The result agreed with those reported by Wang et al., (1997), showing plasmids (pSCV1.6) which have intron gene expression on transferred gene has more efficiency gene expression. The transformation efficiency is comparable with other *Agrobacterium*-mediated transformation systems in monocotyledonous species, such as rice (Aldemita and Hodges, 1996), maize (Ishida *et al.*,1996) and wheat (Hu *et al.*, 2004). This is the first report on *Agrobacterium tumefaciens*-mediated transformation by using the coinfloresence of *C. alismatifolia* Gagnep. An application of this established technique in *C. alismatifolia* Gagnep. will permit the introduction of foreign genes in Curcuma, resulting in crop improvement such as disease resistance, quality of flower colour**.**

In case of *C. alismatifolia* Gagnep. (monocotyledon) had low transformation efficiency level, compared to tobacco and petunia (dicotyledon). This effect might caused by *C. alismatifolia* Gagnep (monocotyledon) was not *Agrobacterium tumefaciens* plants habitat species. That is why the invasion on *C. alismatifolia* Gagnep. of *Agrobacterium tumefaciens* is lower than tobacco and petunia.

CONCLUTIONS

In this study of transformation, *Agrobacterium tumefaciens* strain AGLO harboring the binary vector pSCV1.6 and pBI121 carrying the *uidA* gene encoding GUS activity were chosen to transform into plant tissues of *C. alismatifolia* Gagnep., petunia and tobacco. The transgenic plants were analyzed by detection of histochemical b-glucuronidase (GUS) activity and Polymerase Chain Reaction (PCR). The explants of transformation, *C. alismatifolia* Gagnep for seven months, petunia and tobacco for three months were detected for GUS expression. In case of *C. alismatifolia* Gagnep., only the explants which transformed by pSCV1.6 was detected GUS expression and able to regenerated on selective media. While the GUS expression was found on both of pSCV1.6 and pBI121 in tissues of petunia and tobacco. The efficiency of transformation detected by GUS expression, in case of Curcuma shoots, was 0.83 percent. The explants of petunia and tobacco which transformed with pSCV1.6 showed GUS positive at 23.33 percent and 14.4 percent respectively while the explants of petunia and tobacco showed GUS positive at 13.33 percent and 3 percent respectively when transformed with pBI121.

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