

***Agrobacterium*-mediated Transformation of Mungbean [*Vigna radiata* (L.) Wilczek]**

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ABSTRACT

Plasmid pCAMBIA 1301-choA was transformed into *Agrobacterium rhizogenes* strain K599 and *A. tumefaciens* strain EHA 105 for mungbean transformation. Cotyledons from different ages of mungbean seedlings were inoculated with both bacteria. The two-day-old cotyledons that were co-cultured with hairy root bacteria showed higher ability to produce branched roots than the others. An average of 10 branched roots was formed on both the wounded abaxial site and the hypocotyl cut end. Ten of 75 individual lines (13.25%) showed GUS positive. In addition, cotyledons that were cut and cultured on MB medium supplemented with 2 µgml⁻¹ BAP for 4 days before co-culture with *A. tumefaciens* using hairy root method revealed high transformation ability (31.25%). It was found that seed age, cultivar, co-culture conditions and culture conditions were important factors affecting the transformation efficiency.

Key words : *Agrobacterium rhizogenes* - *Agrobacterium tumefaciens* -
Cholesterol oxidase (*choA*) gene - Insect control protein -
Hairy root - Mungbean transformation

INTRODUCTION

Mungbean [*Vigna radiata* (L.) Wilczek] is an important grain legume in Asia and Thailand. However, the productivity and quality of the grain is severely reduced after harvest as well as during storage by insect infestation. Cowpea weevil (*Callosobruchus maculatus* F.) and soybean weevil (*C. chinensis* L. : Coleoptera: Bruchidae) are the most devastating insects in mungbean (1). As new tools become available, genetic engineering offers an ability to introduce new gene(s) into a crop species. The potential of genetic engineering to incorporate insect resistance into plants has been recognized, for example, in cotton and maize. These genetically engineered crops will reduce the use of chemical insecticides and decrease the exposure of farmers to insecticides as well as the accumulation of chemical residues in the environment.

A novel insecticidal protein that kills boll weevil larvae (*Anthonomus grandis* Bohemam), a key cotton pest, was recently discovered in *Streptomyces* culture filtrates (2). The protein was identified as cholesterol oxidase, a new class of insect control protein. This protein appeared to disrupt the insect gut via the enzymatic oxidation of cholesterol in midgut membranes. Since its discovery, Corbin et al (3) described an expression of cholesterol oxidase gene (*choM*) in tobacco protoplasts. Cho et al (4) succeeded in the expression of the *Streptomyces* sp. SA-COO cholesterol oxidase gene (*choA*) in tobacco (BY-2) by bombardment of tobacco cells with plasmid pBC4. The cholesterol oxidase activity in transformed callus was about eightfold higher than that in control cell line. However, transgenic lines were not described in both experiments. The aim of this study was to design cholesterol oxidase gene suitable for expression in mungbean at an adequate level for control of insect pests. The production of cholesterol oxidase protein in genetically modified mungbean may provide a method of controlling seed-damaging insects that cause severe loss of mungbean in storage.

MATERIALS AND METHODS

Plant Materials

Seeds of mungbean cv. Kamphaeng Saen 1 (KPS 1) and Suranaree 1 (SUT 1) were surface sterilized for 15 min with 20% Clorox before getting rinsed with sterile distilled water. Treated seeds were germinated on MS (5) basal medium containing 3.0% (w/v) sucrose and 0.2% (w/v) Gelrite, pH 5.8. The cultures were incubated at 28°C under 16-h photoperiod (ca 1,500 lx) before being used for transformation.

Bacterial Strains

The plasmid pBC4 carrying cholesterol oxidase (*choA*) gene was obtained from Dr. Yoshikatsu Murooka, Department of Biotechnology, Osaka University, Japan. This plasmid was digested with *Hind*III (Gibco) followed by partial digestion with *Eco*RI to release a 2.8-kb fragment that contained the *choA* gene. The *choA* gene was under the control of cauliflower mosaic virus 35S (CaMV35S) promoter and nopaline synthase polyadenylation (NOS polyA) region. Then the *choA* gene was ligated with the 11.786-kb fragment of pCAMBIA 1301 that comprised T-DNA border flanking chimeric hygromycin phosphotransferase (*hpt*) gene as a selection marker for transformed plant cell, a *gusA* with a catalase intron as the reporter gene and kanamycin resistance as a bacterial selection. The ligated DNA was transformed into *Escherichia coli* DH5 α competent cells by using the heat shock method according to Sambrook et al (6). The vectors pCAMBIA 1301 with inserted *choA* gene were mobilized in *Agrobacterium rhizogenes* strain K599 and *A. tumefaciens* strain EHA 105 by electroporation according to Cho et al (7), and were renamed *A. rhizogenes* K599 pCAMBIA 1301-choA and *A. tumefaciens* EHA 105 pCAMBIA 1301-choA respectively.

Transformation with *A. rhizogenes* K599 pCAMBIA 1301-choA

Cotyledon explants were prepared from the two-, five- and seven-day-old mungbean seedlings cv. KPS 1 by cutting a horizontal slice through the hypocotyl

region. Then a lengthwise slice was cut between the cotyledons, and the embryonic axis removed. This manipulation generated two cotyledonary node explants. An overnight culture of *A. rhizogenes* strain K599 pCAMBIA 1301-choA and the strain lacking this binary vector (a control) were grown on the medium with and without 50 μgml^{-1} kanamycin respectively. Each of the cotyledons was inoculated on the abaxial surface by using a No. 11 scalpel blade previously dipped into the bacterial culture strain being tested. Inoculated cotyledons were then cultured abaxial side up on filter paper immersed in sterile distilled water and incubated at 24°C and 25±3°C in the dark. Three days after inoculation, cotyledons were transferred and cultured in the same manner on MB medium [MS medium with Gamborg's (8) B₅ vitamins] containing 500 μgml^{-1} timentin (ticarcillin disodium, SmithKline Beecham, USA) with or without 25 μgml^{-1} hygromycin and cultured at 28°C and 25±3°C under 16 h light.

Induced roots, 2-cm-long root tips, were excised randomly from each cotyledon and were assumed as one line of transformed root. Two or three induced roots per cotyledon were excised for propagation on the medium supplemented with or without 10, 25 and 40 μgml^{-1} hygromycin. The bacterial decontamination was achieved by two or three subcultures on the same medium at 28°C in the dark. Each subculture was done every 3 weeks. After subculturing twice, histochemical GUS assay of each line was determined and plantlet regeneration was attempted. At the third week of the hairy root growth, 3-cm long root tips were excised and transferred to fresh MB medium with and without growth regulator at 28°C under 16 h light. To assess the influence of growth regulator, 0.1 and 0.5 μgml^{-1} thidiazuron (TDZ), 0.5 and 2.0 μgml^{-1} 6-benzylaminopurine (BAP) or kinetin (KN) and 1.0 μgml^{-1} indole-3-acetic acid (IAA) was supplied singly or in combination in the MB medium.

Transformation with *A. tumefaciens* EHA105 pCAMBIA 1301-choA

The cotyledonary nodes with proximal half of cotyledon attached of one-, two-, three-, and four-day-old seedlings of mungbean cv. KPS1 and SUT1 were excised and pre-incubated before inoculation. The pre-incubation was performed on MB medium containing 2 μgml^{-1} BAP for 3, 4, 5 and 7 days at 25±3°C. The treated cotyledons were inoculated by using two methods. The first method was soybean method whereby the cotyledons were wounded at the cotyledonary node before inoculation with an overnight culture of bacterial suspension and placed in the laminar flow for 30 min. The second method was hairy root method whereby the cotyledons were wounded by making 8-12 slits at the cotyledonary node while being submerged in an overnight culture of bacterial suspension strain that was being tested. The inoculated cotyledonary nodes were co-cultivated on MB medium containing 2, 5 and 10 μgml^{-1} BAP with or without 100 μM acetosyringone in the dark at 24°C and 25±3°C for 3 days. After co-cultivation, the explants were transferred to MB medium supplemented with 2 μgml^{-1} BAP with or without 25 μgml^{-1} hygromycin and 500 μgml^{-1} timentin under 16 h light at 28°C for selection of transformed shoot. Two weeks later, the inoculated explants were assayed for GUS activity.

Histochemical GUS Assay

GUS (β -glucuronidase) activity was determined according to Cho et al (7) with some modifications. Tissues were transferred to a sterilized plastic petri dish containing the GUS assay buffer. This buffer composed of 50 mM sodium phosphate

(pH 7.0), 0.04% (w/v) X-gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) and 0.3% (v/v) Triton X-100. The tissues were incubated overnight at 37°C, cleared in 95% ethanol, and subsequently incubated at 37°C every 3 h twice prior to visual observation.

RESULTS AND DISCUSSION

A. Transformation with *A. rhizogenes* pCAMBIA 1301-choA

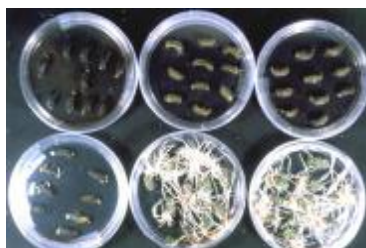
The inoculated cotyledons cultured on selection medium had globular callus developed within 5-7 days on the abaxial site as well as adventitious roots developed without callus formation on the hypocotyl cut end (**Figure 1a**). Root primordia differentiated from callus tissue after culture on the medium without hygromycin in 10-14 days (**Figure 1b**). The medium containing 25 μgml^{-1} hygromycin severely inhibited growth of cotyledons on different days after germination and on various treated cotyledons (**Figure 1c**). This concentration also inhibited roots that developed from both the abaxial side and hypocotyl cut end. There were few small roots developed when hygromycin was present.



(a)



(b)



(c)

Figure 1. Mungbean transformation with *A. rhizogenes*. (a) Callus induction after 7 days of culture. (b) Root primordia differentiated from callus tissue. (c) Hairy root 3 weeks after inoculation. Top row: medium containing 25 μgml^{-1} hygromycin and 500 μgml^{-1} timentin; bottom row: medium without hygromycin (from left to right : control, K599 and K599 pCAMBIA1301-choA).

The ability of root induction decreased progressively when culturing cotyledons were germinated 2, 5 and 7 days on the medium without hygromycin. At age 2 days, germinating cotyledons inoculated with strains K599 and K599 pCAMBIA 1301-choA, showed the highest root production (97.67% and 96.23% respectively) compared to root production at age 5 days (79.38% and 75.47%) and 7 days (50.98% and 42.86%) as shown in **Table 1**. This result was similar to that of Rech et al (9) who reported that explant age influenced *Glycine max* seedlings transformation with *Agrobacterium* strains carrying Ri plasmid. This could be due to the high efficiency of young cells to grow and differentiate. Moreover, these 2-day-old cotyledons inoculated with strain K599 pCAMBIA 1301-choA produced an average of 10 highly branched roots per explant from both the wounded abaxial side and the hypocotyl cut end. The length of the hairy roots was 0.5-7 cm after 21 days of infection. In contrast, 100% of the cotyledons wounded with a sterile scalpel (uninoculated control) produced only one tap root and 2-3 adventitious roots on the hypocotyl cut end (data not shown). Cotyledons inoculated with strain K599 lacking the binary vector produced a small amount of callus and roots on the abaxial side and the hypocotyl cut end.

Table 1. Effect of days after germination and the medium containing 500 μgml^{-1} timentin on root induction.

Treatments	Days after germination (d)	No. of inoculated cotyledon	No. of cotyledon with roots	% of root induction	No. of roots/ Cotyledon
Uninoculated	2	15	15	100	2.34
	5	16	2	12.5	1.45
	7	50	4	8	2.02
K599	2	43	42	97.67	3.69
	5	97	77	79.38	7.41
	7	51	26	50.98	1.92
K599 pCAMBIA 1301-choA	2	53	51	96.23	10.22
	5	106	80	75.47	2.81
	7	105	45	42.86	3.73

A total of 75 out of 83 individual roots that were excised randomly and cultured on MB medium supplemented with 25 μgml^{-1} hygromycin could survive after the fourth subcultures. Most of the roots exhibited rapid growth, formed lateral branches, and grew not only into the solid medium but also upwards. From the total of 75 individual roots, 11 roots (13.25%) were GUS positive (**Figure 2a, b, c**). The intensity of the GUS activity varied from line to line, indicating that there may be either the copy number of inserted genes or the position effects arising from the integration of the gene (10). Although these GUS positive lines could survive on hygromycin selection medium up to 40 μgml^{-1} , they grew slowly with little branching and showed geotropism. In contrast, roots induced from uninoculated or inoculated with strain K599 lacking the binary vector stopped growing and died on the medium containing hygromycin. No GUS activity was observed in these tissues (**Figure 2d**).

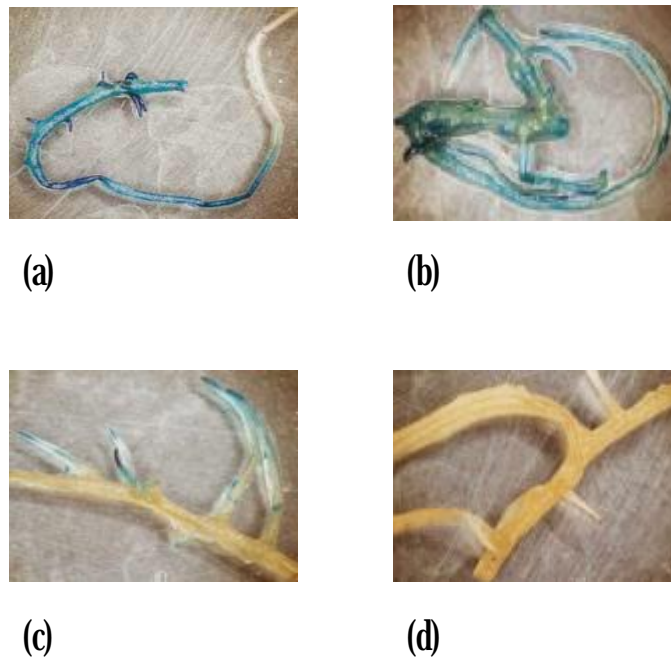


Figure 2. GUS activities of transformed roots (a, b, c) and control (d).

In many plant species including some legumes such as *Astragalus sinicus* (11), alfalfa (12), *Lotus corniculatus* (13) and the wild perennial soybean species *Glycine canescens* (9), spontaneous shoots have been regenerated from the transformed roots after infection with *A. rhizogenes*. On the contrary, many reports have indicated that the addition of growth regulators particularly cytokinins to the culture medium can enhance shoot production from the hairy roots of horseradish (14) and *Glycine argyrea* (15). In mungbean, however, the transformed hairy roots could not spontaneously regenerate. Although growth regulators were added to the MB medium, no transgenic plantlet could be induced from hairy roots in this experiment.

B. Transformation with *A. tumefaciens* pCAMBIA 1301-choA

Cotyledons, pre-incubated on MB medium containing $2 \mu\text{gml}^{-1}$ BAP before inoculation with *A. tumefaciens* EHA105 pCAMBIA 1301-choA, showed high transformation efficiency (**Table 2**). The two-day-old seedlings, pre-incubated for 4 days, showed the highest ability for transformation (31.25%). Thirty-nine cotyledonary nodes were inoculated, but only 32 nodes were assayed for GUS activity after culture for two weeks. The results showed that 10 out of 32 cotyledonary nodes had GUS positive activity with 1-3 GUS blue sectors per explant (**Figure 3a, b, c and d**). One emerged shoot from these GUS-positive cotyledons showed GUS activity on its shoot and leaf (**Figure 3e**). However, the cotyledonary nodes of one- or two-day-old seedlings, pre-incubated for one, two, and seven days before inoculation with a bacterium lacking the binary vector and uninoculated controls, showed GUS negative

results. In soybean, Santarem et al (16) indicated that there was no beneficial effect of pre-incubation on transient transformation efficiency. However, they suggested that the effects of pre-incubation needed to be re-evaluated for stable transformation work as the mitotic state of the target tissue might have different effects on transient and stable transformation. In the present experiment, the pre-incubation for four days was more responsive to transformation than others.

Table 2. Effect of pre-incubation on cotyledon transformation using hairy root method.

Treatments	Seedling ages	Pre-incubation (days)	No. of inoculated cotyledon	No. of cotyledon showing GUS positive
Control	1	1	20	0
		2	22	0
	2	4	38	0
		7	40	0
EHA 105	1	1	21	0
		2	19	0
	2	4	39	0
		7	38	0
EHA 105 pCAMBIA 1301-choA	1	1	65	0
		2	60	0
	2	4	39	10
		7	37	0

In this experiment, two co-cultures and culture conditions were used, at 25°C and 25±3°C. It was observed that these factors had influence on transformation efficiency. This result was similar to that of Kudirka et al (17) who found that stem segments of soybean infected with *Agrobacterium* and co-cultured at 25°C enhanced transient expression while higher temperature suppressed the transfer of T-DNA. Fullner and Nester (18) also examined the effect of temperature on the efficiency of T-DNA transfer. Mungbean cultivars could also affect transformation efficiency. In this study, the best Thai cultivars, KPS1 and SUT1, were used and differential response was observed. In India, Jaiwal et al (19) reported the success of mungbean transformation from seven commercial mungbean cultivars.

Furthermore, the presence of acetosyringone during co-cultivation tended to enhance transformation efficiency. In azuki bean (20), the addition of acetosyringone alone had no obvious effect on transient GUS expression but high concentration of BAP up to 10 µgml⁻¹ with 100 µM acetosyringone caused efficient gene transfer by *Agrobacterium*. On the contrary, this study showed that 5 or 10 µgml⁻¹ BAP with 100 µM acetosyringone could produce more multiple shoots with fast growth, but GUS activity was not present. Thus acetosyringone did not result in high rate of transformation. Similar results in peanut were also reported by Mansur et al (21) and Cheng et al (22)

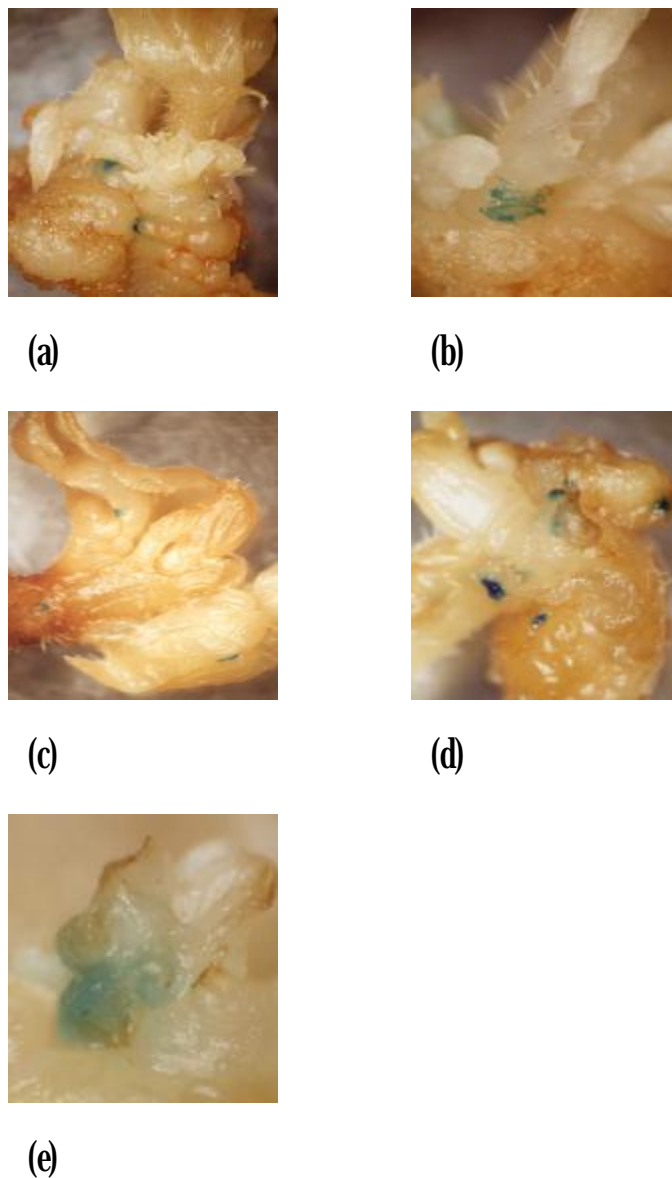


Figure 3. Cotyledonary node transformation with *A. tumefaciens*. Inoculated cotyledons showed GUS positive (a, b, c, d) and shoot emerged from a GUS positive cotyledon (e).

CONCLUSIONS

This experiment focused on using cotyledonary nodes or cotyledons as explants for *Agrobacterium*-mediated transformation. By using this system, 31.25% transformation was obtained, but only one GUS-positive shoot could be regenerated.

Transformation methods also influenced the production of transformed plant. With soybean method, explants did not show GUS activity compared to using hairy root method. In addition, the latter method was less laborious and less exposed to bacterial contamination compared to the previously reported protocol. The new method also gave high percentage of hairy root induction. This study could induce 42.86%-96.23% hairy roots on the wounded surface of the cotyledons. On the other hand, only 13.25% of the lines was GUS positive. Not only the wounding technique had influence on transformation, but the cultivars, seed age, co-culture conditions, and culture conditions were also important factors affecting the transformation efficiency.

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บทคัดย่อ

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การใช้อะโกรแบคทีเรียเพื่อการถ่ายยีนเข้าสู่ถั่วเขียว [*Vigna radiata* (L.) Wilczek]

พลาสมิด pCAMBIA 1301-choA ได้ถูกถ่ายเข้าไปใน *Agrobacterium rhizogenes* สายพันธุ์ K599 และ *A. tumefaciens* สายพันธุ์ EHA105 เพื่อใช้ในการถ่ายยีนเข้าสู่ถั่วเขียว ใบเลี้ยงถั่วเขียวจากต้นกล้าอายุต่างๆ ถูกนำมาใช้เลี้ยงร่วมกับเชื้อทั้งสองสายพันธุ์ พบว่าใบเลี้ยงอายุ 2 วัน ที่เลี้ยงร่วมกับเชื้อสายพันธุ์ที่ชักนำให้เกิดรากแสดงความสามารถในการสร้างรากฝอยจำนวนมากกว่าใบเลี้ยงอายุอื่น ๆ โดยเฉลี่ยเกิดรากฝอย 10 ราก ทั้งบริเวณรอยกรีดด้านบน และรอยตัดที่ไฮโปคอตลิล โดยรากฝอย 10 ราก จาก 75 ราก (13.25 เปอร์เซ็นต์) แสดงกิจกรรมของยีน *gus* ส่วนใบเลี้ยงที่ตัดและเลี้ยงบนอาหาร MB ที่เติม BAP เข้มข้น 2 ไมโครกรัมต่อมิลลิเมตร เป็นเวลา 4 วัน ก่อนนำไปเลี้ยงร่วมกับเชื้อ *A. tumefaciens* โดยใช้วิธี hairy root ให้ประสิทธิภาพในการถ่ายยีนสูง (31.25 เปอร์เซ็นต์) การศึกษานี้พบว่า อายุเมล็ด พันธุ์ สภาพแวดล้อมในการเลี้ยงรวม และสภาพแวดล้อมที่วางเลี้ยง เป็นปัจจัยที่สำคัญที่มีผลต่อประสิทธิภาพการถ่ายยีน

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