INTRODUCTION AND EXPRESSION OF CHOLESTEROL OXIDASE GENE IN A BACTERIUM [*Escherichia coli* M15 (pREP4)] AND MUNGBEAN [*Vigna radiata* (L.) Wilczek]

Potjamarn Suraninpong

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การถ่ายยีน และการแสดงออกของยีนโคเลสเตอรอลออกซิเดส ในแบคทีเรีย [*Escherichia coli* M15 (pREP4)] และ ในถั่วเขียว [*Vigna radiata* (L.) Wilczek]

พจมาลย์ สุรนิลพงศ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Thesis Examining Committee

.....
(Sopone Wongkaew, Ph.D.)
Chairman

.....
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พจมาลย์ สุรนิลพงศ์ : การถ่ายยีนและการแสดงออกของยีนโคเลสเตอรอลออกซิเดสในแบคทีเรีย [*Escherichia coli* M15 (pREP4)] และในถั่วเขียว [*Vigna radiata* (L.) Wilczek] (INTRODUCTION AND EXPRESSION OF CHOLESTEROL OXIDASE GENE IN A BACTERIUM [*Escherichia coli* M15 (pREP4)] AND MUNGBEAN [*Vigna radiata* (L.) Wilczek]

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การทดลองนี้มีวัตถุประสงค์เพื่อถ่ายพลาสมิด pCAMBIA 1301 ที่สร้างขึ้น ซึ่งมียีนโคเลสเตอรอลออกซิเดส (*choA*) เชื่อมต่ออยู่ (pCAMBIA 1301-choA) เข้าสู่ *Agrobacterium rhizogenes* สายพันธุ์ K599 และ *A. tumefaciens* สายพันธุ์ EHA 105 และใช้สำหรับถ่ายยีนเข้าสู่ถั่วเขียว โดยใช้ใบเลี้ยงอายุต่าง ๆ ไฮโปคอททิล และใบอ่อนของต้นกล้าถั่วเขียวพันธุ์ กำแพงแสน 1 (KPS 1) และ มทส. 1 (SUT 1) การชักนำให้เกิดต้นที่สมบูรณ์จากรากที่ได้รับการถ่ายยีน ทำการศึกษาบนอาหารที่มีและไม่มีสารควบคุมการเจริญเติบโต นอกจากนี้ยังได้ศึกษาการโคลนนิ่ง การแสดงออก และการสกัดโปรตีนโคเลสเตอรอลออกซิเดส ที่ถูกตัดแต่งพันธุกรรมใน *E. coli* M15 (pREP4) ในการศึกษาครั้งนี้ยีนโคเลสเตอรอลออกซิเดส (*choA* or *choa*) จาก *Streptomyces* ที่อยู่ในพลาสมิด pCO117 ถูกตัด และนำไปเชื่อมต่อกับเวกเตอร์ pQE 30, pQE 31 and pQE 32 ก่อนทำการนำเข้าสู่ *E. coli* M15 (pREP4)

ผลการทดลองแสดงให้เห็นว่าใบเลี้ยงของถั่วเขียวพันธุ์ กำแพงแสน 1 อายุ 2 วัน หลังจากเพาะเมล็ด ซึ่งเลี้ยงร่วมกับ *A. rhizogenes* K599 pCAMBIA 1301-choA มีความสามารถในการสร้างรากฝอยได้สูงกว่า (96.23 %) ใบเลี้ยงอายุ 5 และ 7 วัน หลังจากเพาะเมล็ด (75.47 % และ 42.86 %, ตามลำดับ) โดยเฉลี่ยแล้วสามารถชักนำให้เกิดรากฝอยประมาณ 10 ราก ต่อใบเลี้ยงทั้งบนหลังใบที่ทำแผล และบริเวณไฮโปคอทิลที่ถูกตัด รากฝอยจำนวน 11 ราก จากจำนวน 75 ราก(13.25 %) แสดงกิจกรรมของยีน *gus* การชักนำพืชที่สมบูรณ์จากรากเหล่านี้ไม่ประสบผลสำเร็จ ข้อใบเลี้ยงอายุ 2 วัน ของสายพันธุ์กำแพงแสน 1 และ มทส. 1 ที่วางเลี้ยงเป็นเวลา 4 วัน ก่อนเลี้ยงร่วมกับ *A. tumefaciens* EHA 105 pCAMBIA 1301-choA แสดงประสิทธิภาพในการถ่ายยีนที่สูง (31.25 %) อย่างไรก็ตาม การถ่ายยีนเข้าสู่ถั่วเขียวในการศึกษานี้ยังไม่ประสบผล

นอกจากนี้พบว่า เวกเตอร์ pQE 30 เมื่อเชื่อมต่อกับยีน *choA* or *choa* ให้ลำดับเบสที่สามารถแปลรหัสได้ แต่ไม่พบกิจกรรมของเอนไซม์โคเลสเตอรอลออกซิเดส จากเวกเตอร์ pQE30-choa การตรวจสอบที่อยู่ของโปรตีนจากสายพันธุ์ pQE 30-choA ซึ่งแสดงออกใน *E. coli* M15 (pREP4) เป็นโปรตีนที่อยู่ภายในเซลล์แบคทีเรีย แต่โปรตีนที่ไม่บริสุทธิ์จากแบคทีเรีย M15 (pREP4) pQE 30-choA แสดงกิจกรรมของเอนไซม์ต่ำกว่าแบคทีเรียหน่วย การทดลองเปรียบเทียบ M15 (pREP4) pCO117 (317 กับ 560 มิลลิยูนิต ต่อ มิลลิกรัมโปรตีน ตามลำดับ) การวิเคราะห์ระยะเวลาที่เหมาะสมต่อการแสดงออกของโปรตีนของ M15 (pREP4) pQE 30-choA แสดงให้เห็นว่ามีกิจกรรมของเอนไซม์สูงสุดหลังจากเลี้ยงแบคทีเรียเป็นเวลา 4 ชั่วโมงในอาหารที่มี IPTG โปรตีนที่สกัดจากสายพันธุ์ M15 (pREP4) pQE 30-choA มี 1 แบน มีกิจกรรมของเอนไซม์ 1.2 ยูนิต ต่อ มิลลิกรัมโปรตีน โปรตีนมีน้ำหนักโมเลกุล 57 กิโลดาลตัน เท่ากับที่วัดได้จากสายพันธุ์ M15 (pREP4) pCO117 แต่แตกต่างจากโปรตีนโคเรสเตอรอลออกซิเดส ของ *Streptomyces* ที่ผลิตจำหน่ายโดยบริษัท Sigma ประเทศอเมริกา (55 กิโลดาลตัน) แม้ว่าได้ทำการศึกษาอายุของแบคทีเรีย อาหารเลี้ยงเชื้อ การใส่เอนไซม์ที่ยับยั้งการย่อยโปรตีน อุณหภูมิ ความหนาแน่นของเซลล์ และความเข้มข้นของ IPTG แต่ไม่ทำให้กิจกรรมของเอนไซม์สูงขึ้น เพราะสายพันธุ์แบคทีเรียที่สร้างขึ้นมีกรดอะมิโนเกินมา 8 ตัว, มี *lac* operon 2 ตำแหน่ง มีลำดับเบส ATG เริ่มต้นทั้งของเวกเตอร์และของยีน *choA* และมีลำดับเบส Shine-Dalgarno ที่ *E. coli* ชอบ ระบบนี้ไม่เหมาะสมต่อการแสดงออกของยีนโคเลสเตอรอลออกซิเดส ที่ผลิตจาก *Streptomyces*

สาขาวิชาเทคโนโลยีการผลิตพืช **ลายมือชื่ออาจารย์ที่ปรึกษา.....**

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ลายมือชื่อนักศึกษา..... **ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....**

POTJAMARN SURANINPONG : INTRODUCTION AND EXPRESSION OF CHOLESTEROL OXIDASE GENE IN A BACTERIUM [*Escherichia coli* M15 (pREP4)] AND MUNGBEAN [*Vigna radiata* (L.) Wilczek]
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This experiment was attempted to transform the constructed plasmid pCAMBIA 1301 with a cholesterol oxidase gene (*choA*) insertion (pCAMBIA 1301-choA) into *Agrobacterium rhizogenes* strain K599 and *A. tumefaciens* strain EHA 105 and used for mungbean transformation. Cotyledons of different ages, hypocotyl and young leaves of mungbean cv. KPS 1 and SUT 1 seedlings were inoculated with both bacteria. Regeneration of the transformed root was studied on media with and without growth regulators. In addition, the cloning, expression and purification of recombinant cholesterol oxidase protein in *Escherichia coli* (*E. coli*) M15 (pREP4) were studied. The *Streptomyces* cholesterol oxidase gene (*choA* or *choa*) from plasmid pCO117 was cut and ligated with the expression vectors pQE 30, pQE 31 and pQE 32 before transformation into *E. coli* M15 (pREP4).

The result showed that the cotyledons of mungbean cv. KPS 1 two days after germination that were co-cultured with *A. rhizogenes* K599 pCAMBIA 1301-choA showed higher ability to produce branched roots (96.23 %) than did the cotyledons of 5 and 7 days after germination (75.47 % and 42.86 %, respectively). An average of 10 branched roots per cotyledon were formed on both the wounded abaxial side and the hypocotyl cut end. Eleven out of 75 individual roots (13.25 %) were GUS positive. The roots could not be regenerated into plants. The two-day-old cotyledonary node of cv. KPS 1 and SUT 1 that were pre-incubated for 4 days before co-culture with *A. tumefaciens* EHA 105 pCAMBIA 1301-choA using hairy root method revealed high transformation ability (31.25 %). However, mungbean transformation in this study was unsuccessful.

In addition, it was found that the vector pQE 30 when ligated with *choA* or *choa* gene revealed the correct reading frame. However, the enzyme activity could not be detected in the clone carrying vector pQE 30-choa. The target protein from clone pQE 30-choA that expressed in *E. coli* M15 (pREP4) was an intracellular solubility. Crude cell lysate of clone M15 (pREP4) pQE 30-choA showed lower enzyme activity (317 mU mg protein^{-1}) than did the control clone M15 (pREP4) pCO117 (560 mU mg protein^{-1}). Time course analysis of the M15 (pREP4) pQE 30-choA showed the highest activity (350 mU mg protein^{-1}) after the cells were cultured for 4 h with isopropyl-β-D-thigalactoside (IPTG). Purified protein from clone M15 (pREP4) pQE 30-choA showed single band with enzyme specific activity of 1.2 U mg protein^{-1}. The molecular weight of this protein was 57 kDa, the same as that detected from M15 (pREP4) pCO117 but different from a commercial *Streptomyces* cholesterol oxidase (Sigma USA, 55 kDa). Although inoculum age, medium, proteinase inhibitor, temperature, density of cells and IPTG concentrations were examined, the enzyme activity was not increased. The fact that the constructed clone had 8 extra amino acids, two *lac* operon, ATG initiation codon from both the vector and the *choA* gene, and Shine-Dalgarno sequence favored by *E. coli*, made this system unsuitable for the expression of *Streptomyces* cholesterol oxidase gene.

School of Crop Production Technology
Academic Year 2002
Student. Potjamarn Suraninpong

Advisor. Aree Waranyuwat
Co-advisor. .....
Co-advisor. .....

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LIST OF ABBREVIATIONS

A	=	Absorbance
Act-2-T	=	actin terminator
amp^R	=	ampicillin resistance gene
BAP	=	6-benzylaminopurine
bar	=	phosphinothricin acetyltransferase gene
B_5	=	Gamborg *et al* (1968) medium
$CaMV_{35}S$	=	cauliflower mosaic virus 35S promoter
CaMV35S polyA	=	cauliflower mosaic virus 35S polyadenylation region
choA	=	*Streptomyces* sp. A19249 cholesterol oxidase gene
choB	=	*Brevibacterium sterolicum* cholesterol oxidase gene
choM	=	*Streptomyces* sp. SA-COO cholesterol oxidase gene
choP	=	sequence encoding cytochrome P450-like protein
Da	=	Dalton
GUS	=	β-glucuronidase
hpt	=	hygromycin phosphotransferase
IAA	=	indole-3-acetic acid
IPTG	=	isopropyl-β-D-thiogalactoside
LB	=	Lauria broth
kDa	=	Kilo Dalton
KPS 1	=	Kamphaeng Saen 1
KN	=	kinetin
M	=	molarity
MB	=	MS basal medium plus B5's vitamin
min	=	minute
MS	=	Murashige and Skoog (1962) medium
mU mg $protein^{-1}$	=	milliunit per miligram protein
mM	=	milli molar
NAA	=	α-naphthaleneacetic acid

LIST OF ABBREVIATIONS (continued)

nm	=	nanometer
NOS poly A	=	nopaline synthase polyadenylation region
*npt*II	=	neomycin phosphotransferase gene
OD	=	Absorbance
pH	=	-log hydrogen ion concentration
PMSF	=	phenyl methyl sulfonyl fluoride
rpm	=	revolution per minute
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SUT 1	=	Suranaree 1
TDZ	=	thidiazuron
Ubi-3-P	=	ubiquitin promoter
Ubi-3-T	=	ubiquitin terminator
X-gluc	=	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
°C	=	degree Celsius
μg ml^{-1}	=	microgram per milliliter
μl	=	microliter
μM	=	micro molar
% (v/v)	=	percent volume per volume
% (w/v)	=	percent weight per volume

CHAPTER I

INTRODUCTION

Mungbean [*Vigna radiata* (L.) Wilczek] is an important grain legume in Asia. Its seed is a good source of proteins and other essential nutrients. However, yield and quality of the grain is severely reduced by insect infestation before and after harvesting. Cowpea weevil, *Callosobruchus maculatus* (F.) and soybean weevil, *C. chinensis* (L.) (Coleoptera: Bruchidae) are the most serious insects infesting on mungbean (Sarikarin *et al*., 1999). A warm and humid climate in Asia favors quicker insecticide degradation as well as rapid pest population build-up (Talekar and Chen, 1983). Therefore, the need to control insect pests in mungbean will always be necessary.

Recently, a novel insecticidal protein that kills boll weevil (*Anthonomus grandis grandis* Boheman) larvae, a key cotton pest, was discovered in *Streptomyces* culture filtrates (Purcell *et al*., 1993). The protein was identified as cholesterol oxidase and it is a new kind of insect control protein. Although this protein has shown toxicity to some insects, the potential use to control pests in crops has not been demonstrated.

The aim of this study is to design plasmids containing cholesterol oxidase gene for expression in mungbean which would eventually be useful for controlling seed-damaging insects that cause severe loss to mungbean in storage. An efficient production of cholesterol oxidase enzyme in a bacterium *Escherichia coli* could

facilitate the large-scale production of the cholesterol oxidase protein for use in industry.

The objectives of this study are :

1. To construct the plasmid containing *choA* gene for use in mungbean transformation.
2. To demonstrate the expression of *Streptomyces* cholesterol oxidase gene in *E. coli*.

CHAPTER II

REVIEW OF LITERATURE

1. *Vigna* and *Phaseolus* species

The importance of grain legumes is a protein source for consumption. Species of both genera *Vigna* and *Phaseolus* are widely grown throughout the world, particularly in Asia and South America. Similarities of *Vigna* and *Phaseolus* species have been confused for many years. Recently, the detailed molecular analysis represents the relationship of these two genera. The most important taxa in *Vigna* species include *V. unguiculata*, also known as cowpea and black eyed pea, *V. radiata*, also known as mungbean and *V. aconitifolia*, also known as moth bean. The major cultivated representative in genus *Phaseolus* is *P. vulgaris*, also know as French bean, haricot bean and kidney bean. The other important beans in this group include *Vicia faba* (Windsor or broad bean), *P. limensis* (lima bean), *P. coccineus* and *Vigna radiata* (mungbean) (Christou, 1992). Since, leguminous plants in general, and *Vigna* and *Phaseolus* species in particular, are somewhat recalcitrant and difficult to regenerate *in vitro* (Widholm, 1995). There has been quite a lot of research activities going on in the recent past in order to regenerate and to genetically transform *Vigna* and *Phaseolus* species (Nagl *et al*., 1997).

2. *Agrobacterium*

Plant transformation has been successful in many species, such as rice (Alam *et al*., 1999), corn (Gordon-Kamm *et al*., 1990), cotton (Perlak *et al*., 1990) and citrus (Hidaka *et al*., 1990). *Agrobacterium*-mediated transformation has been the best method available for DNA transfer to tissue explants (Hinchee *et al*., 1998). This technique utilizes *Agrobacterium* as a biological vector to introduce a portion of its DNA into the plant genome, resulting in production of transformed plant (Nagl *et al*., 1997). The two species of *Agrobacterium* normally used for this purpose have different characteristics. *A. tumefaciens*, the soil-borne pathogen causes crown gall disease and *A. rhizogenes*, on the other hand, produces hairy roots. Crown gall disease and hairy root induction is due to the integration of T-DNA into the plant genome and its subsequent expression. *A. tumefaciens* with a disarmed Ti-plasmid can introduce foreign genes into plant cells without altering their capacity to regenerate whole plant *in vitro* (Puonti-Kaerlas *et al*., 1990). Genes encoded by the T-DNA cause the overproduction of auxin and cytokinin, which leads to the proliferation of gall or tumor tissue (Godwin *et al*., 1991), whereas the T-DNA of *A. rhizogenes* consists of the non-continuous TL (left) and TR (right) regions of the root inducing (Ri) plasmid of the bacterium. Ri transformed roots are characterized by their ability to synthesize opines and to proliferate, with extensive lateral branching, on culture medium lacking growth regulators (Kumar *et al*., 1991). Thus, this bacterium would be useful for studying mungbean transformation. The study of *Vigna* and *Phaseolus* species transformation with *Agrobacterium* is reviewed in the following sections.

3. *Agrobacterium tumefaciens*-mediated gene transfer in *Vigna*

Garcia *et al*. (1986, 1987) have reported the successful and stable introduction of foreign genes into *V. unguiculata* tissues using *A. tumefaciens* strain harboring a Ti-plasmid which contained two copies of kanamycin resistance gene or a full-length DNA copy of cowpea mosaic virus. The kanamycin resistant callus was obtained. Whole plants were not recovered from transformed cells, but Southern and Northern blot analysis of the transformed callus confirmed the integration and full-length transcripton of those gene. Penza *et al*. (1991) tried to transform mature embryos of *V. unguiculata* with *A. tumefaciens* A281.They obtained chimeric transgenic calli, but no plants could be regenerated from the calli. Hence stable integration of foreign gene could not be conclusively proven. However, Kononowicz *et al*. (1993) reported the production of chimeric-transgenic plants of *V. unguiculata* under kanamycin selection using both *A. tumefaciens* and particle bombardment systems. Nagl and Ehemann (1994) reported the transient and stable expression of GUS gene in calli obtained from transformed protoplasts of *V. unguiculata* ssp. *sequipedalis*. However, plant regeneration from transgenic calli was unsuccessful. Muthukumar *et al*. (1996) described the conditions for effective transformation and regeneration of cowpea using cotyledon with intact proximal end. Cotyledon pre-incubated on B5 basal medium supplemented with 8 μM BAP for 24 h before co-cultured for 48 h, subsequently transferred to shoot induction medium containing 25 μg ml^{-1} hygromycin, enhanced the transformation efficiency. The presence of hygromycin phosphotransferase (*hpt*) gene in the transgenic plants was confirmed by Southern blot hybridization.

Eapen *et al*. (1987) reported the effect of genotype on *V. aconitifolia* transformation. A large number of kanamycin-resistant colonies were obtained from

protoplasts co-cultivated with *A. tumefaciens*. Their result showed that cultivar was an important factor in achieving high transformation frequencies. The moth bean cv. IPCMO-560 displayed 85-fold higher transformation rate than cv. IPCMO-909. Although transformed plants were reported, no molecular or genetic analysis was demonstrated of their transgenic plants. In species *V. mungo*, Karthikeyan *et al*. (1996) obtained transgenic calli by co-cultivating segments of primary leaves with *A. tumefaciens*. Transformed calli were found to be resistant to kanamycin up to 900 μg ml^{-1} concentration. The stable integration of transformed cell was confirmed by Southern blot analysis.

Recently, Yamada *et al*. (2001) reported the successful transformation of azuki bean, *V. angularis*. Seedling epicotyl explants were co-cultured with *A. tumefaciens* for 2 days on MS medium supplemented with 100 μM acetosyringone and 10 μg ml^{-1}BAP. The plantlet was obtained within 5-7 months after co-cultivation. Polymerase chain reaction (PCR) and Southern blot analysis confirmed stable integration and expression of foreign genes. Jaiwal *et al*. (2001) reported the successful transformation of *V. radiata* by using four-day-old cotyledonary node. The explants which were pre-inoculated in bacterial suspension for 6 h followed by co-cultivation under dark conditions at 25±2°C for 3 days showed the highest transient transformation frequency. Southern blot analysis confirmed the integration and expression of transgene (*gusA* and *npt*II) in T_0 plants and seeds.

4. *Agrobacterium tumefaciens*-mediated gene transfer in *Phaseolus*

Mariotti *et al*. (1989) transformed *P. vulgaris* and *P. coccinneus* with *Agrobacterium* strain LBA 4404 harboring the vector pBI121. Gene transfer was

achieved by co-cultivation of shoot apexes that were cut at the level of primary leaf node with bacterial suspension. They had some plants which resisted to kanamycin and expressed GUS gene activity. Franklin *et al*. (1993) obtained kanamycin-resistant callus from leaf disc or hypocotyl explants of *P. vulgaris* when cultured on medium supplemented with kanamycin after co-cultivation with *Agrobacterium*. Bean callus cultures were also transformed with chalcone synthase promoter GUS fusion. These cultures, when treated with the elicitor glutathione, showed higher levels of GUS expression than the unelicited callus. Lewis and Bliss (1994) stab-inoculated intact shoot tips of germinating seed of ten *P. vulgaris* lines with *Agrobaterium*. Tumor formation and GUS gene expression was obtained in the meristem region, but transformed shoot was unobtained. Becker *et al*. (1994) transformed cotyledonary nodes and leaf nodes of different cultivars of *P. vulgaris* with *A. tumefacies* A281. They found that the selection medium strongly influenced the further growth of explants. Kanamycin at 50 μg ml^{-1} inhibited growth of the explants within three weeks and at 100 and 200 μg ml^{-1} the explants could survive for two and one week, respectively.

5. *Agrobacterium rhizogenes*-mediated transformation in legume

Manners and Way (1989) investigated the potential of *A. rhizogenes* as a helper for transformation of a pasture legume *Stylosanthes humilis*. They found that stem co-cultured with *Agrobacterium* in the dark for 3 days was the best condition on transformation. This condition also yielded kanamycin resistant root at a frequency up to 86 % and subsequent plant regeneration at a frequency of 23 %. However, abnormal plants induced from transformed root were observed. Rech *et al*. (1989) described the

production of transgenic plants of *Glycine canescens* using an engineered strain of *A. rhizogenes* (R1601) carrying a chimeric *npt*II gene. The hypocotyls excised from various seedling ages were inoculated with this *Agrobacterium* and the transformed roots were regenerated on B5 medium with 10 μg ml^{-1} BAP, 0.05 μg ml^{-1} IBA and 50 μg ml^{-1} kanamycin. In the study, they suggested that transformation with *Agrobacterium* be influenced by explant age and a much higher concentration of BAP was required to induce plant regeneration. Judith-Webb *et al*. (1990) had established hairy root from three species of legume, *Lotus corniculatus*, *Trifolium repens* and *T. pratence*, by using a wild-type (C581) strain of *A. rhizogenes*. Southern blot analysis confirmed that the lines were genetically transformed and the copy number of TL-DNA in different lines varied from one to eight. The transformed root revealed changes in anatomy, morphology and cytology, but plants obtained from hairy roots of *L. corniculatus* showed no change in several parameters of nitrogen fixation.

Damiani and Arcioni (1991) reported the successfully transformed *Medicago arborea*, a shrubby pasture crop for dry land, with *A. rhizogenes*. The introgression of an antibiotic resistance gene (hygromycin) identified the optimal method for genetic transformation, as well as to introduce a marker gene for *in vitro* selection of hybrid cell produced by protoplast fusion. Kumar *et al*. (1991) demonstrated the stable integration of wild type and genetically engineered Ri T-DNA into the genome of wild soybean, *G. argyrea*, and its expression. This paper also emphasized the effect of seedling age and bacterial concentration on transformation frequency. Thus hypocotyls from young seedlings were the most responsive in producing roots at inoculation sites. A suspension of 1.0 x 10^7 bacteria ml^{-1} was optimal for strain LBA9402, whereas 1.0 x 10^8 bacteria ml^{-1} were the most effective with strains A4T, R1601 and A4TIII.

Transgenic plants were confirmed by the presence of opines and by dot blot analysis for Ri TL-DNA. Cho *et al*. (1998) reported the establishment of a transformation and regeneration system for *Astragalus sinicus* (a winter growing green manure legume) by infection with *A. rhizogenes* harboring both pRi1724 and a second binary vector, pBI121. Transformed plants regenerated from cultured roots was confirmed by histochemical GUS activity and Southern blot analysis. Recently, Cho *et al*. (2000) described a new high-efficiency hairy root transformation system for soybean by infection cotyledon with *A. rhizogenes* strain K599 harboring both Ri-and the binary vectors, pBI121 or pBINm-gfp5-ER. The hairy roots were produced 54-95 % from the wounded surface of cotyledon explants. However, inducing shoot formation from the transformed hairy roots failed.

6. Insect control with genetically engineered crops

To date insect resistance genes transferred into plants mainly target to the insect digestive system. Most have been derived from microorganisms (*Bacillus thuringiensis* and *Streptomyces*) or a range of higher plants. Much work throughout the world is devoted to obtain insecticidal genes from plants of different species. These genes that show resistance to insect pests include lectins, α-amylase, chitinase and trytophan decarboxylase, collectively called proteinase inhibitors. This part of the review will present insect resistance genes that have been successfully used.

6.1 The use of proteinase inhibitors

Plant proteinase inhibitors are polypeptides or proteins that occur naturally in a wide range of plants and are a part of the plant's natural defense system against

herbivors (Schuler *et al*., 1998). These molecules interfere the growth and development of the larvae and in some cases cause the death of the insect (Jovanin *et al*., 1998). According to their specificity, proteinase inhibitors can be divided into four classes; e.g. inhibiting serine, cystein (or thiol), metallo and aspartyl proteinases. These proteinases catalyze the release of amino acids from dietary protein and so provide the nutrients crucial for normal growth and development (Schuler *et al*., 1998). The antimetabolic mode of action of these inhibitors is not fully understood. Direct inhibitors of digestive enzyme are not considered to be the main effect, the important factor might be the hypersecretion of digestive enzymes caused by the presence of the inhibitors, resulting in depletion of essential amino acids. However, some insect species seem to be able to adapt their enzyme activity by switching to a proteinase not affected by the inhibitors. Gut digestive enzymes also affect the water balance, moulding and enzyme regulation of the insects (Schuler *et al*., 1998).

At present, at least 14 different plant proteinase inhibitor genes have been introduced into crop plants. Most effort has concentrated on serine proteinase inhibitors from plant families *Fabaceae*, *Solanaceae* and *Poaceae*, which target mainly against lepidopteran, some coleopteran and orthopteran pests.

6.2 The use of lectins

Lectins are carbohydrate binding proteins found in many plant tissues, and are abundant in the seed and storage tissues of some plant species. Various lectins have shown some toxic activity against the insect orders Homoptera, Coleoptera, Lepidoptera and Diptera (Schuler *et al*., 1998). The mode of action of lectins against insects remains unclear but it has been shown that at least some bind to midgut

epithelial cells. However, some insecticidal lectins also show toxicity to mammalians, including lectins from *Phaseolus vulgaris*, *Psophocarpus tetragonobus* (winged bean), soybean and wheat germ. This toxicity will limit their potential use in the production of transgenic crop plants. Recently, interest has mainly concentrated on the lectin from snowdrop (*Galanthus niralis*; GNA) or garlic because it has shown activity against aphids and the rice brown planthopper (*Nilaparvala tugens*), but does not exhibit mammalian toxicity (Gatehouse and Hilder, 1994; Jovanin *et al*., 1998). GNA has been expressed in nine different crops, including potato, oilseed rape and tomato. Tobacco plants expressing a pea lectin are shown to be toxic to the Lepidoptera, *Heliothis virescens*, and potato plant expressing the snowdrop lectin is toxic to the Lepidoptera, *Lacanobia oleracea*. Most work involving lectins has focused on the obtention of aphid resistant plants. To date, no evaluation of the long term interest of this strategy has been published (Gatehouse and Hilder, 1994; Gatehouse *et al*., 1993; Greenplate *et al*., 1995; Jovanin *et al*., 1998).

6.3 The use of α-amylase

The α-amylase inhibitor (αAI) has been described in the seeds of several varieties of the common bean, *Phaseolus vulgaris*, and in cereal endosperm of wheat (Greenplate *et al*., 1995). This inhibitor is the second of enzyme inhibitors used to modify crop plants. Genes for three αAI have been expressed in tobacco, but the emphasis has been on transferring the gene for the common bean (αAI-Pv) to other legumes. These inhibitors are toxic to insect and mammalian but not active against plant and bacterial α-amylase. αAI-Pv inhibits α-amylase in the midgut of coleopteran stored-product pests of bruchid beetles, *Callosobruchus maculatus*, *C. chinensis* and

Bruchus pisorum. The transfer of this αAI gene to Azuki bean conferred resistance to three species of bruchids. However, it is difficult to evaluate the long term interest of the expression of this gene in plants.

6.4 The use of tryptophan decarboxylase

Tryptophan decarboxylase (TDC) from *Catharanthus roseus* (L.) (periwinkle) converts tryptophan to indole-alkaloid tryptamine. Tryptamine is thought to participate in the protection of young seedling against insects, particularly in newly-emerged seedling stems (Thomas *et al*., 1995). The TDC gene when expressed in transgenic tobacco reduces the reproduction of the whitefly (*Bemisis tabaci*) by up to 97 %. Because the production of tryptamine, its derivatives or other products resulting from TDC activity may discourage whitefly reproduction and provide single gene based plant protection strategy. No papers report the long term expression of this gene in plant and its application.

6.5 The use of *Bacillus thuringiensis* (Bt) δ-endotoxin

Bacillus thuringiensis is a common soil bacterium that produces insecticidal protein crytals called *Bt* toxin, δ-endotoxin or crystal protein within its cells during the sporulation process. This toxin when consumed by the insect is hydrolyzed in the gut to release toxic protein fragments. The basis of toxicity is thought to be a disruption of gut membrane function mediated through specific receptor site in the brush border of the midgut epithelial cells (Gatehouse and Hilder, 1994 and Peferon, 1997).

Each strain of the bacteria produces different crystal proteins. At least ten genes encoding different Bt toxins have been engineered into plants. Each of the crystal proteins has a specific activity spectrum. For example, the *Cry* 1Ab protein is highly active against Lepidoptera, the European corn borer, and is currently used in Bt corn hybrids (Boulter, 1993; Gatehouse *et al*., 1993). The *Cry* 1Ac protein is highly toxic to both tobacco budworm and cotton bollworm larvae and is expressed in the *Bt* cotton varieties commercialized in the USA and Australia, while the *Cry* 3A protein is expressed in *Bt* potato varieties and provides protection from Colorado potato beetles (Peferon, 1997).

6.6 The use of cholesterol oxidase

The enzyme cholesterol oxidase was discovered in *Streptomyces* culture filtrates represents a new class of insect control protein. Cholesterol oxidase (cholesterol : O2 oxidoreductose, EC 1.1.3.6) is a bifunctional enzyme that catalyzes the oxidation of cholesterol (5-cholesten-3-β-ol) to the temporary intermediates 5-cholesten-3-one with the reduction of oxygen to hydrogen peroxidase (Allain *et al*., 1974). Cholesterol oxidase has strong insecticidal activity against boll weevil larvae (*A. grandis grandis* Boheman) (Purcell *et al*., 1993). The activity of the cholesterol oxidase protein is in the range of the *B. thuringiensis* δ-endotoxin and has an LC_{50} of 20.9 μg ml^{-1} against the boll weevil. Diet incorporation and histological studies of the boll weevil midgut epithelium indicate the mode of action of the cholesterol oxidase as a direct disruption of the midgut cells, rather than an effect related to the enzyme altering critical diet and nutritional requirements. So cholesterol oxidase can act in two ways in transgenic plants to reduce boll weevil population through a reduction of adult

fecundity as well as through lethal and developmental effects on larvae. Although the lethal and developmental effects of cholesterol oxidase on boll weevil are similar to the effects of *Bt* toxins on their respective insect targets, the molecular mechanisms by which the two types of proteins act are very different. Cholesterol oxidase appears to disrupt the insect gut via the enzymatic oxidation of cholesterol in the midgut membranes. This differs from the mechanism of action of *Bt* toxin, which binds to specific midgut membranes. Because of this difference, cholesterol oxidase represents a new class of insect control protein. Although cholesterol is a fundamental component of the membranes of all cells within the animal kingdom, the United States Department of Agriculture, the Environmental Protection Agency, and the Food and Drug Administration suggest that plants containing cholesterol oxidase will be safe for human and animal consumption. This enzyme, like most other proteins, is denatured and inactivated by heating, so processed cotton seed or products of that kind consumed by humans would not contain active enzyme. Another suggestion is the enzyme structure different from known toxins or allergens and it is rapidly digested by gastric fluids of the kind found in mammalian digestive tracts. The digestive conditions in the upper gastrointestinal tract of ruminants are robust, it appears that cholesterol oxidase will not pose a risk to these animals (Cho *et al*., 1995; Greenplate *et al*., 1995; Perferon, 1997).

7. Cloning and expression of *Streptomyces* cholesterol oxidase

Since Allain *et al*. (1974) first illustrated the suitability of cholesterol oxidase for the analysis of total serum cholesterol, cholesterol oxidase has been the most widely used in clinical assays. Cholesterol oxidase (E.C 1.1.3.6) catalyzes the

oxidation of cholesterol (5-cholesten-3-β-ol) to the intermediates 4-cholesten-3-one with the reduction of oxygen to hydrogen peroxide (Murooka *et al*. 1986; Ishizaki *et al*., 1989; Nomura *et al*., 1995).

Cholesterol oxidase has been found in culture broth of *Streptomyces* species and other microorganisms such as *Brevibacterium sterolicum* (Uwajima *et al*., 1973), *Streptoverticllium cholesterolicum* (Inouye *et al*., 1982), *Schizophyllum commune* (Fukuya and Miyake, 1979; Kenney *et al*., 1979), *Rhodococus* sp. (Johnson and Somkuti, 1991) and *Pseudomonas* sp. (Lee *et al*., 1989). Because of its commercial value, there is widespread interest in producing this enzyme in high yields through genetic engineering. Thus, this review discusses cholesterol oxidase in the topics: (1) cloning and expression of *Streptomyces* cholesterol oxidase in *Streptomyces*, *Escherichia coli* and lactic acid bacteria, such as *Bacillus* spp. and *Lactobacillus*, (2) cloning and expression of *Brevibacterium* cholesterol oxidase in *E. coli*, (3) cholesterol oxidase purified from the other sources, and (4) application of *Streptomyces* cholesterol oxidase as a potent insecticidal protein.

7.1 Cloning and expression of *Streptomyces* cholesterol oxidase in *Streptomyces*, *Escherichia coli* and lactic acid bacteria

Sterptomyces species produce a large variety of medical enzyme in the culture broth and are attractive as a host for expression of useful genes by recombinant DNA. Murooka *et al*. (1986) were interested in the control of secretion and in the mechanism of action of cholesterol oxidase enzyme from *Streptomyces*. So, they cloned the cholesterol oxidase (*cho*) gene from *Streptomyces* sp. strain SA-COO into *Streptomyces lividans* 1326 and *S. griseus* KSN with a multicopy vector pIJ 702.

A *cho* gene that is different in size was ligated with vector pIJ 702 and renamed as pCO-1, pCO-2 and pCO-3 before being transformed into *S. lividans* and *S. griseus* KSN. Cell of *S. lividans* and *S. griseus* KSN carrying those plasmids had an ability to produce cholesterol oxidase. The highest yield of the enzyme was obtained from *S. lividans* cells carrying pCO-3 grown in GMP medium. The level of extracellular and intracellular cholesterol oxidase was two-fold and 13-fold over that detected in original strain SA-COO, respectively. Approximately 40 % cholesterol oxidase produced from this clone was secreted into the culture broth and was stably produced at least for 8 days. The yield of an intracellular and extracellular enzyme were about 4.8 and 3.1 U mg^{-1} of cells, respectively. Moreover, deletion analysis of the recombinant plasmid showed that the size of the DNA fragment essential for the cholesterol oxidase production was within 2.5 kb, which allows for a protein of about 91,000 daltons. Normally, *Streptomyces* species produce extracellular cholesterol oxidase, but the ability to secrete in the host cells is probably insufficient. Thus, they concluded that the secretion system in *Streptomyces* may be useful for the production of genetically engineered proteins.

In 1989, Ishizaki *et al*. described the complete nucleotide sequence and the amino acid analysis of extracellular cholesterol oxidase from *Streptomyces* strain SA-COO. Their studies showed that *choA* was a structural gene for *Streptomyces* cholesterol oxidase. The complete nucleotide sequence of this gene is 2,143 base pairs, which consists of an open reading frame of 1,638 base pairs, the GTG initiation codon at position 399 and the TAA termination codon at position 2038. The signal peptide is extended over 42 amino acids. Thus, the mature enzyme contains 504 amino acids with molecular weight of 54,913. Horii *et al*. (1990) determined nucleotide sequence

of the *choA* gene promoter region. The *choA* gene consists of operon with cytochrome P-450 like protein (*choP*). So, the promoter region for *choP* is essential for an expression of the *choA* gene. A 2.9 kilobase transcript resulted from Northern blot analysis showed the identical size to the total sequence of the *choP* and *choA* gene. These two genes are transcribed polycistronically under the control of promoter upstream of *choP*. The *choP* gene encodes 381 amino acids with a molecular weight of 41,668. Since, no endogenous activity of cholesterol oxidase was found in any enteric bacteria, Nomura *et al*. (1995) modified cholesterol oxidase as a reporter gene by constructing convenient promoter-prove vectors that can be used in enteric bacteria. They were successful in expressing the *Streptomyces* cholesterol oxidase gene in *E. coli* by alteration of the promoter and the ribosomal binding sequence, the initiation codon and the 5′ region of the original *choA* gene of *Streptomyces*. The modification of the cholesterol oxidase gene (choA′) was expressed in *E. coli* under the control of the *lac* or *tac* promoter. However, no expression of the native *choA* gene from *Streptomyces* was observed in *E. coli*. The whole regoin of *choP* and a part of the 5′ end of *choA* of *Streptomyces* were deleted and a DNA fragment corresponding to the NH_2-terminus of the *Streptomyce*s enzyme was synthesized. The synthetic 292-bp oligonucleotide fragment was designed for codons that were favorable in *E. coli*, including the ATG initiation codon instead of the original GTG. The synthetic *choA′* gene was ligated with vector pKK223-3 resulting in plasmid pCO117. *E. coli* JM109 carrying the plasmid pCO117, that under the control of *tac* promoter, produced 2-fold more intracellular cholesterol oxidase during 18-h culture than did the original strain of *Streptomyce*s sp. SA-COO cultured for 4 days. The cholesterol oxidase produced by *E. coli* JM109 (pCO117) contained additional 22 amino acid residues in the NH_2-

terminus with the molecular mass of approximately 57 kDa. The NH_2-terminus sequence of amino acids was found to be processed between Ala^{20} and Ala^{21}, while the *Streptomyces* enzyme was processed between Ala^{42} and Asp^{43}. The specific activities of purified cholesterol oxidase from *E. coli* and *Streptomyces* were 23.0 U and 23.5 U mg^{-1} protein, respectively.

Because the efficiency of expression and secretion of cholesterol oxidase was considerably low, to produce the protein stably and in a large amount, Solaiman and Somkuti (1991) were able to subclone and expressed *choA* gene from S*treptomyces* in *E. coli*. Expression of the *choA* gene was observed from plasmid pUC0192A and pUC0193 which the cloned genes were aligned upstream *lac*Z promoter. Specific cholesterol oxidase activity of pUC0193 intracellular with and without isopropyl-β-D-thioglucopyranoside (IPTG) induction was 0.004 U and 0.007 U mg^{-1} protein, respectively. The incomplete expression of cholesterol oxidase production in the absence of IPTG was described as the presence of unidentified substance in the complex LB medium, whereas the cholesterol oxidase activity detected in the medium could suggest the possible secretion of the enzyme. They also concluded that *lac*Z promoter/operator region of pUC19 was needed for the expression of the cloned *choA* gene in *E. coli*. Potentially high total yields and secretion of cholesterol oxidase production from pUC0193-containing *E. coli* transformant was an attractive candidate for further development into a cholesterol oxidase production strain.

It may be of great interest to study the lowering of cholesterol content of foods, in particular fermented dairy products. Cholesterol degradation in milk and food rich in cholesterol by means of cholesterol oxidase has been reported in

Streptococus thermophilus and *Lactobacillus casei* (Somkuti *et al*. 1991, 1992). In 1993, Brigidi *et al* reported on cloning and expression of the *choA* gene from *Streptomyces* sp in *Bacillus*, *Lactobacillus reuteri* and *E. coli*. Cell extracts of *E. coli* transformants harboring pCHOA that contains *choA* gene from *Streptomyces* sp. showed good cholesterol oxidase production (7.3-10.2 mU mg^{-1} protein). In pCHOA with the *lac* promoter of pUC 19 located upstream of the cloned fragment, the addition of IPTG greatly increased the enzyme synthetie rate, reaching the highest activity 2 h after the induction. Without inducer adding, the pattern of cholesterol oxidase production correlated to the cell growth. No enzyme cholesterol oxidase activity was detected in culture broth, because *E. coli* failed to recognize the *Streptomyces* sp. cholesterol oxidase signal peptide sequence. Moreover, they ligated the shuttle vectors containing the replication functions of gram-positive bacteria and selection markers functional in those hosts with pCHOA resulting in pLDA. This plasmid was transformed into *E. coli* HB 101, *B. subtilis* PB 1424, *B. licheniformis* 5A24 and *Lac. reuteri* DSM 20016. In these gram-positive transformants obtained, the cholesterol oxidase gene was stably maintained, but no enzyme activity was exhibited. The failure of gene expression in gram-positive bacteria may be due to the unutilized promoter of the *Streptomyces* in these hosts.

7.2 Cloning and expression of *Brevibacterium* cholesterol oxidase in *E. coli*

Fujishiro *et al*. (1990) isolated cholesterol oxidase gene (CHOD) from *Brevibacterium sterolicum* ATCC 21387 and compared amino acid sequence with cholesterol oxidase (*choA*) from *Streptomyces* sp. Their result showed that the amino acid sequence of *Brevibacterium* enzyme had 53 % resemblance with the

Streptomyces enzyme, but had difference in the middle part. After screening *B. sterolicum* gene library with synthetic probe, they found that the fragment p4-22-15 ligated with pUC 19 contained the CHOD gene**.** This plasmid was transformed to express in *E. coli* MM 294, but no CHOD activity was shown. It was the result of rearrangement occurred and deletion in the promoter or the NH2-terminal protein coding region of CHOD during the construction. Moreover, this strain harboring plasmid pSCA No.8 did not express CHOD activity nor made the extra proteins which resulted from transcription failure. So, they were trying to express the CHOD gene in different hosts, such as *Corynbacterium glutamicum*, *S. lividans* and *B. sterolicum*, but the data were not given.

In 1991 and 1992, Ohta *et al*. determined nucleotide sequence of *B. sterolicum* ATCC 21387 cholesterol oxidase or *choB* gene. The *choB* sequence contained an open reading frame with G+C content of 64.9 mol % that would encode a protein of 552 amino acids. Comparison of the nucleotide sequence of the *choB* gene and deduced amino acid sequence to those of the cho-encoding gene (*choA*) from *Streptomyces* strain SA-COO showed identities of 64% and 58%, respectively. N-terminal amino acid analysis of *B. sterolicum* extracellular cholesterol oxidase confirmed that the mature enzyme consisted of 507 amino acids with molecular weight of 54.902 Da. The expression of *choB* in *E. coli* using *B. sterolicum* promoter gave lower yield of active protein than utilizing the *lac* promoter. However, the protein was expressed as insoluble inclusion bodies. Thus, Sampson and Chen (1998) improved expression of *choB* gene in *E. coli* by utilizing the T7*lac* promoter and modified the gene to encode the first 21 amino acids. These changes resulted in 60-fold improvement of expression level. The recombinant *E. coli* produced protein

composing of 513 amino acids with molecular weight of 55,374 Da. The optimal induction time was determined to be 10 h and purified protein showed specific activity of 44.5 U mg^{-1} protein.

7.3 Cholesterol oxidase purified from other sources

Doukya and Aono (1998) purified extracellular cholesterol oxidase from *Pseudomonas* sp. strain ST-200 and examined the involvement of this activity. The result showed that cholesterol oxidase had a molecular weight of 60 kDa and stabilize at pH 4 to 11, active at pH 5.8 to 8.5, optimal activity shown at pH 7 at 60°C. The rate of ST-200 cholesterol oxidase production was enhanced 3-to 3.5-fold in the presence of organic solvent, such as benzene, toluene, β-xylene, propylbenzene and diphenylmethane, but the oxidation rate was extremely low when cholesterol dissolved in chloroform. On the contrary, the cholesterol oxidation rates of *Streptomyces* sp. and *Brevibacterium* sp. were not enhanced by the organic solvents, as well as the cholesterol oxidation rates of *Nocardia erythropolis* and other *Pseudomonas* sp. were not so high in the presence of organic solvents.

Yazdi *et al*. (2001) purified extracellular cholesterol oxidase from *Streptomyces fradiae* PTCC 1121. The purified enzyme had a molecular weight of 60 kDa and the optimum pH and temperature for activity was found to be 7 and 70°C, respectively. The cholesterol oxidase was stable in pH between 4-10 at 4°C for 4 h. It showed high thermal stability and retained its full activity at 50°C for 90 min.

7.4 Application of *Streptomyces* cholesterol oxidase as a potent insecticidal protein in transgenic plants

Since its discovery, the proteins that control insects were studied. Purcell *et al*. (1993) found that cholesterol oxidase purified from culture filtrate of *Streptomyces* was active against cotton boll weevil larvae (*Anthononus grandis grandis* Bohemam). This protein appeared to disrupt the insect gut via the enzymatic oxidation of cholesterol in midgut membranes. Corbin *et al.(*1994) cloned and sequenced structural cholesterol oxidase gene (*choM*) from *Streptomyces* sp. strain A19249. Isolated genomic DNA from strain A19249 was hybridized with probes N2 and C2, an internal peptide of cholesterol oxidase. The 2.2-kb fragment, which was homologous to the region of probe, was designated as cholesterol oxidase *choM* gene. The *choM* gene was subcloned into bacterial expression vector pKK 233-2 carrying *trc* promoter and a bacterial ribosome binding site. Thus, the recombinant plasmid showed expression in the presence of a *lac* operon inducer by IPTG. The primary translation production of *choM* was predicted to be a 547-amino acid and the first 43 amino acids was constitute as a secretory signal peptide. The purified cholesterol oxidase had a specific activity of 29 U mg^{-1} protein with molecular weight of 54 kDa. Finally, they concluded that expression of the gene with the signal sequence of *E. coli* resulted in the production of a protein that had enzymatic and insecticidal property. Moreover, they first described an expression of cholesterol oxidase gene in plant cell. The cholesterol oxidase (*choM*) gene from *Streptomyces* sp. strain A19249 was electroporated into tobacco protoplasts by using vector pMON11772. The *choM* gene was under the control of figwort mosaic virus promoter and nopaline synthase gene from *A. tumefaciens*. In three independent electroporation experiments, cholesterol oxidase activity of full-length gene was two-

to five-fold higher than that of the mature form of the gene. Thus, the structure of engineered *choM* gene appeared to have an effect on the production of recombinant protein in tobacco protoplasts. An expression of this gene in plant protoplasts was confirmed by Western blot analysis. Cho *et al*. (1995) later succeeded in the expression of the *Streptomyces* sp. SA-COO cholesterol oxidase gene (*choA*) in tobacco (BY-2) cells. A plasmid pBC4 containing the *choA* gene under the control of cauliflower mosaic virus 35S promoter was introduced into tobacco cells by using particle bombardment. The cholesterol oxidase activity of the transformed callus was about 8 folds higher than that of the control cell line. Stable integration of the *choA* gene in the genome of transformed callus line was confirmed by Southern blot analysis. Northern blot analysis confirmed transcription size of the *choA* gene in the transformed cells. However, transgenic plants were not described in both experiments. Recently, Corbin *et al*. (1998) were characterizing the expression and biological activity of *choM* gene produced in transgenic cotton plants.

CHAPTER III

MATERIALS AND METHODS

1. Mungbean transformation

1.1 Plasmid construction for transformation

A. Bacterial strians and culture condition

The *Escherichia coli* DH5α carrying plasmid pBC4 that contains the cholesterol oxidase (*choA*) gene was kindly provided by Dr. Yoshikatsu Murooka, Department of Biotechnology, Osaka University, Japan. The DH5α carrying binary vector pCAMBIA 1301 and pCAMBIA 3301 (CAMBIA International Research and Education Organization, Canberra, Australia) was used as a vector for *Agrobacterium*-mediated transformation. The DH5α carrying plasmid GUSpAPCH7 obtained from Dr. Niu Dong and Dr. Jack M. Widholm, Department of Crop Sciences, University of Illinois, Urbana, IL, USA was used as a vector for bombardment transformation. The *E. coli* DH5α (BRL Life Technologies, Gaithersburg, MD, USA), *Agrobacterium rhizogenes* strain K599 and *A. tumefaciens* strain EHA 105 were used as a host cell.

The plasmid pBC4 contains a cholesterol oxidase (*choA*) gene, a β–glucuronidase (*gus*) gene as a reporter and a neomycin phosphotransferase (*npt*II) gene as a selectable marker. These genes are under the control of cauliflower mosaic virus 35S (CaMV35S) promoter and nopaline synthase polyadenylation (NOS polyA)

region. This plasmid contains an ampicillin resistance gene (amp^R) as a bacterial selection (Figure 1).

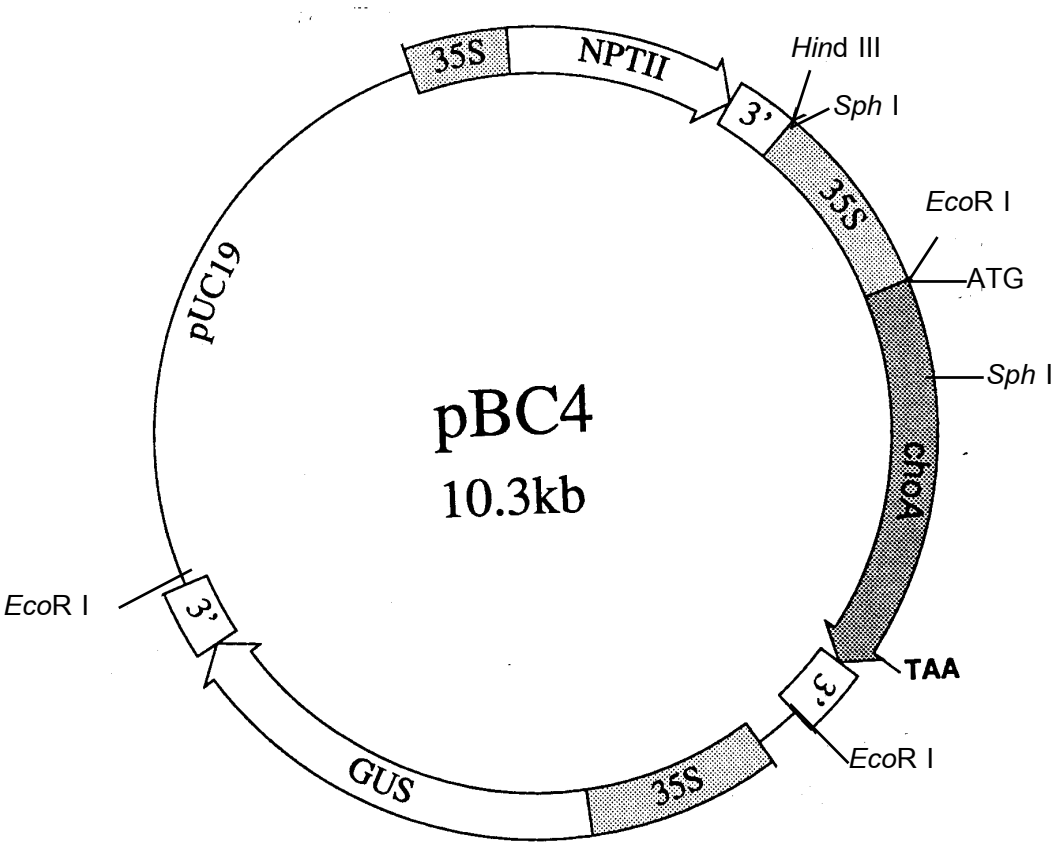


Figure 1. Schematic representation of plasmid pBC4.

The binary vector pCAMBIA 1301 and pCAMBIA 3301 comprised T-DNA borders flanked by chimeric hygromycin phosphotransferase (*hpt*) gene and phosphinothricin acetyltransferase (*bar*) gene, respectively, as a selection marker for transformed plant cell and both have a *gus*A with a catalase intron as the reporter gene and kanamycin resistance gene as a bacterial selection. The *gus* gene is under the control of CaMV35S promoter and NOS polyA region, the *hpt* gene and *bar* gene are under the control of CaMV35S promoter and CaMV35S polyadenylation (CaMV35S polyA) region (Figure 2 and 3).

Plasmid GUSpAPCH7 has a *gus* intron gene as a reporter, *hpt* as a plant selection and an amp^R gene as a bacterial selection. The *gus* gene is under the control of double CaMV35S promoter and an actin terminator (Act-2-T). The *hpt* gene is under the control of ubiquitin promoter (Ubi-3-P) and ubiquitin terminator (Ubi-3-T) (Figure 4).

The *E. coli* DH5α carrying plasmid pBC4 and GUSpAPCH7 was cultured in both liquid and solid LB medium containing 100 μg ml^{-1} ampicillin (Sigma), but the bacteria harboring the plasmids pCAMBIA 1301 and pCAMBIA 3301 were grown in the same medium and supplemented with 50 μg ml^{-1} kanamycin (Sigma). These bacteria were cultured at 37°C. The *Agrobacterium* of both strains, *A. rhizogenes* K599 and *A. tumefaciens* EHA 105, were cultured separately in the same medium without antibiotics, but the constructed *A. rhizogenes* K599 and *A. tumefaciens* EHA 105 carrying plasmid pCAMBIA 1301 or pCAMBIA 3301 were cultured in a medium containing 50 μg ml^{-1} kanamycin at 28°C.

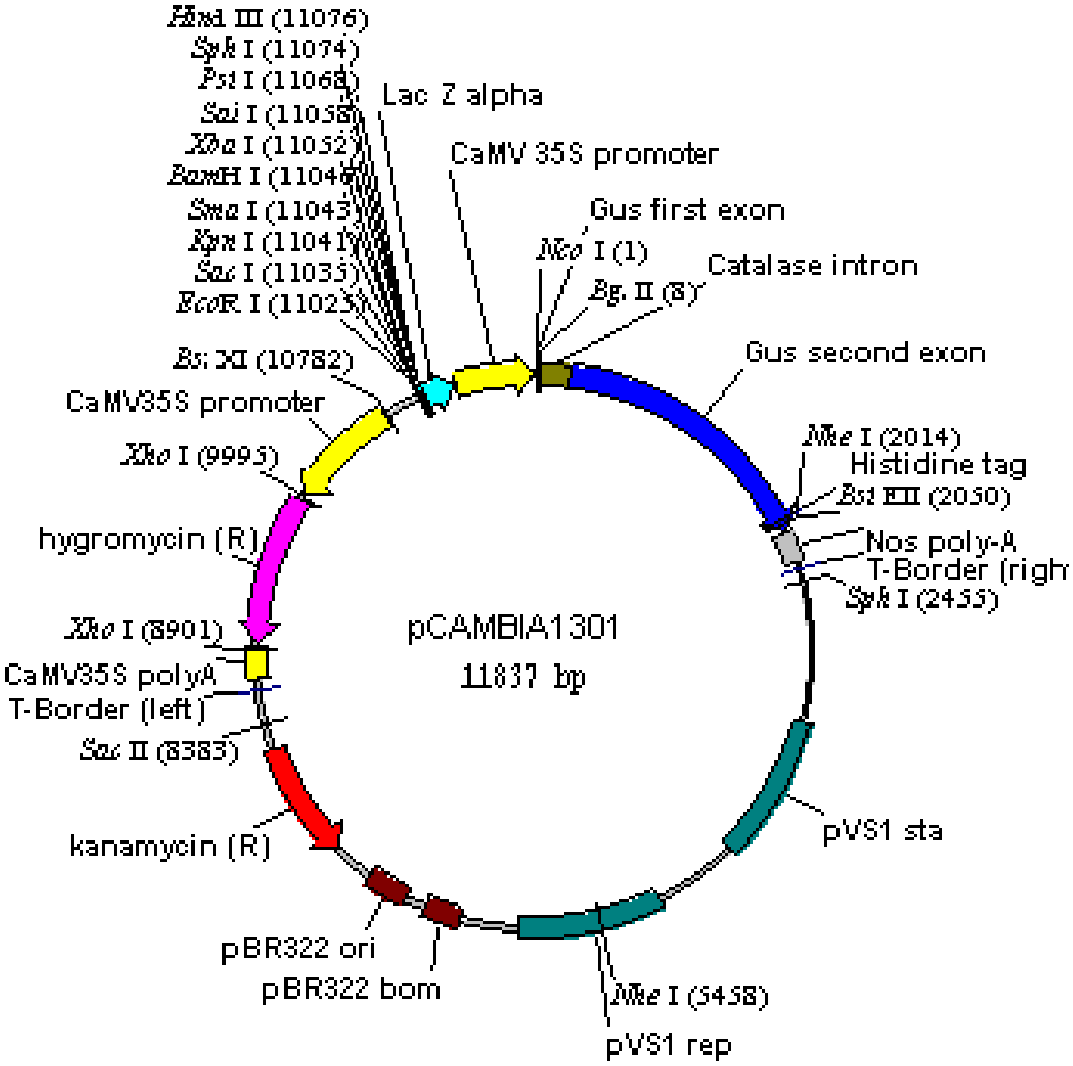


Figure 2. Schematic representation of binary vector pCAMBIA 1301

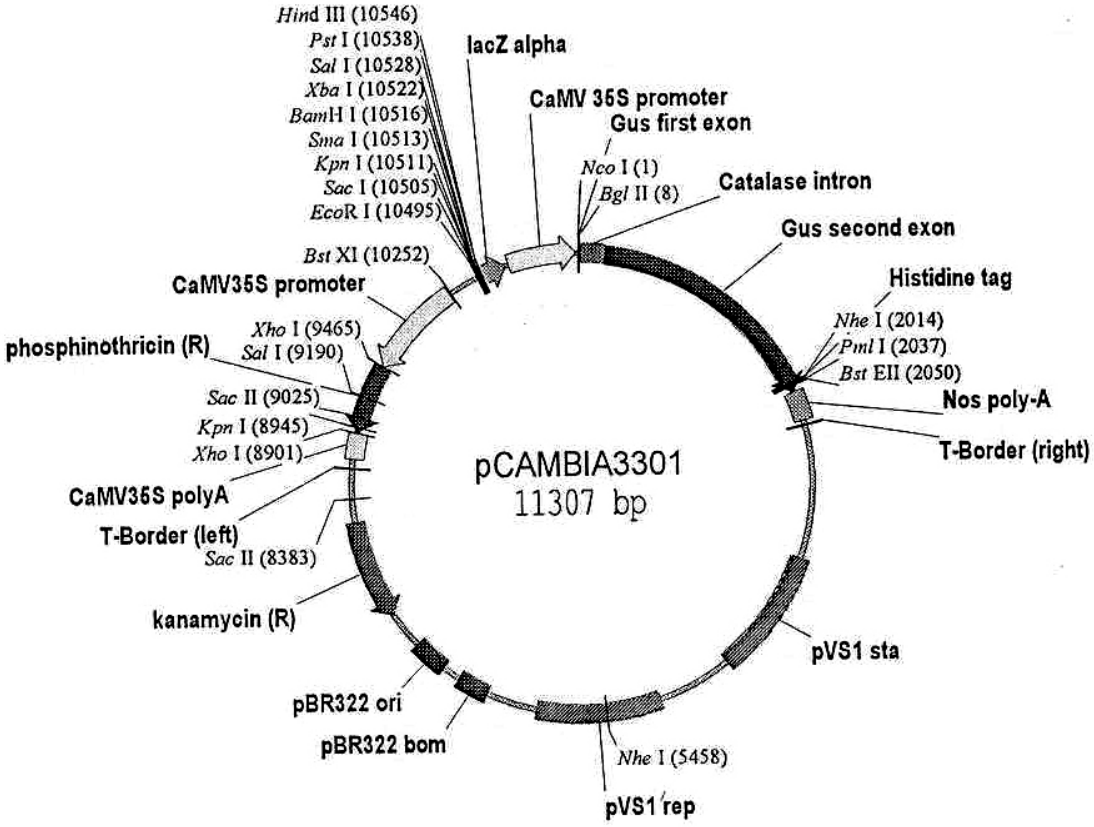


Figure 3. Schematic representation of binary vector pCAMBIA 3301

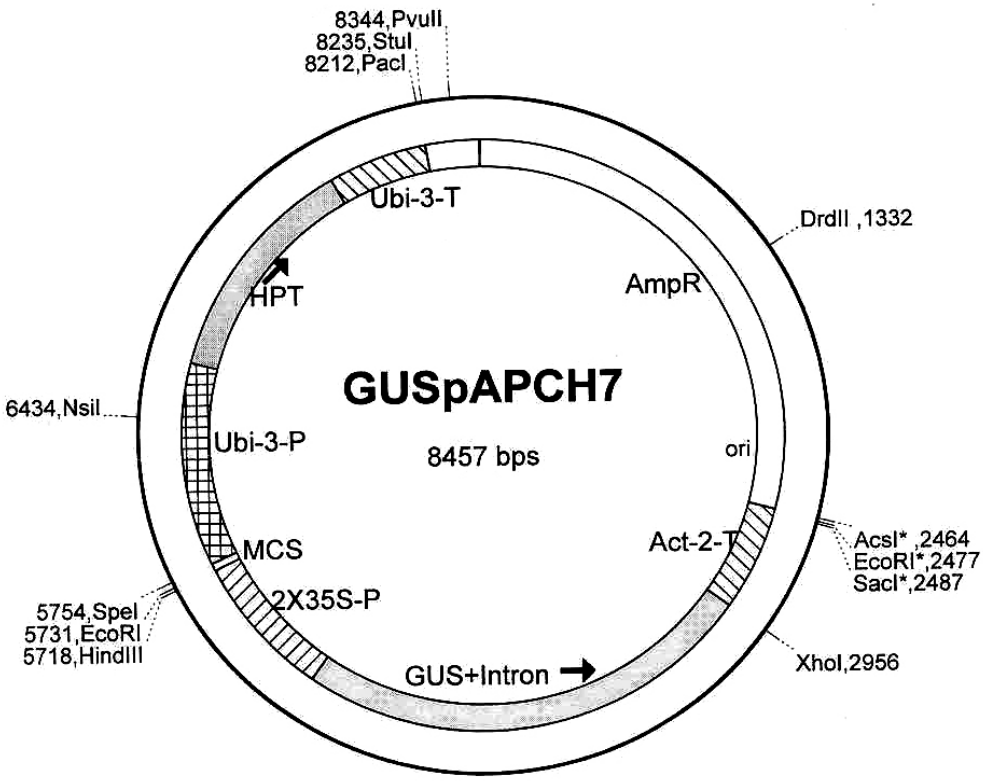


Figure 3. Schematic representation of GUSpAPCH7

B. DNA preparations

The DNA extraction from *E. coli* DH5α carrying plasmid pBC4, pCAMBIA 1301 or pCAMBIA 3301 was prepared by using QIAGEN plasmid Mini kit (QIAGEN, USA). The DNA extraction from *Agrobacterium* of both strains, K599 and EHA 105, carrying constructed plasmid pCAMBIA 1301 or pCAMBIA 3301 with the *choA* gene insertion was conducted according to Cho *et al*. (2000) with slight modification by using P1, P2 and P3 buffer from QIAGEN plasmid Mini kit. After adding the P3 buffer, the solution was incubated on ice for 30 min. Then the solution was added with chloroform : isoamyl at 24 : 1 ratio and mixed gently before centrifugation at 14,000 rpm for 10 min at 4°C. The supernatant was added with 1 ml

of 100 % icy cold ethanol and kept at –70°C for 15 min before centrifugation at 14,000 rpm for 10 min at 4°C. The DNA pellet was washed with 70% ethanol, dried 3 min under vacuum, dissolved with sterile distilled water and kept at –20°C before use.

Prior to subcloning, the plasmid DNA was cut with appropriate restriction enzymes and fractionated on 0.8 % agarose gel (Seakem® LE Agarose, FMC BioProducts, USA). The electrophoresis was performed by using EASY-CAST™ Electrophoresis System Model # 82 (OWL Scientific Inc, USA) at 80-120 volts. The specific DNA fragments were recovered and purified by Geneclean II kit (BIO 101 Inc, USA).

C. Preparation of competent cells

The competent cell of the *E. coli* DH5α and the *Agrobacterium* strains K599 and EHA105 were prepared according to Sambrook *et al*. (2000) and Cho *et al* (1998), respectively. For preparation of *E. coli* DH5α competent cell, a single colony of the bacteria was cultured overnight at 37°C before inoculation into 100 ml SOB medium. After an absorbance$_{595}$ of the bacterial suspension was reached 0.4-0.6, the suspension was kept on ice for 15 min followed by centrifugation at 3,500 rpm, 4°C for 15 min. Then, the pellet was gently re-suspended with 33 ml of icy cold filter-sterilized RF1 before centrifugation at the same condition. Before using, the pellet was gently re-suspended with 2 ml of icy cold filter-sterilized RF2, divided 100 µl in an aliquot and kept at –80°C.

For preparation of *Agrobacterium* competent cell, a single colony of the bacteria was cultured for 2 days at 28°C before inoculation into 100 ml LB broth

medium. After overnight cultured at the same conditions, the bacterial suspension was kept on ice for 10 min and harvested by centrifugation at 6,000 rpm, 4°C for 10 min. The pellet was re-suspended in 100, 50, 2 and 1 ml of filter-sterilized glycerol followed by centrifugation at the same conditions. The cells were divided into 40 µl in aliquots and kept at –80°C before use.

D. Plasmid transformation and selection

The ligation product of linear fragment of pCAMBIA 1301 or pCAMBIA 3301 and GUSpAPCH7 with *choA* gene was transformed into *E. coli* DH5α by heat-shock procedure according to Sambrook *et al*. (2000) for multiplication. The transformed cells were plated on LB medium supplemented with 50 µg ml^{-1} kanamycin and 100 µg ml^{-1} ampicillin. The surviving colonies were picked randomly and DNA extracted from selected clone was analyzed using restriction enzymes. Thus, the extracted plasmid pCAMBIA 1301 or pCAMBIA 3301 that has a *choA* insertion from *E. coli* DH5α was transformed into *Agrobacterium* strains K599 and EHA105 competent cells by using electroporation according to Cho *et al* (1998). While the plasmid GUSpAPCH7 with *choA* insertion was transformed into *E. coli* DH5α competent cells by using heat-shock procedure according to Sambrook *et al*. (2000).

E. Preparation of bacterial suspension for transformation

A single colony of *A.rhizogenes* K599, *A. tumefaciens* EHA105 carrying plasmid pCAMBIA 1301-choA and the bacteria of both strains carrying no plasmid (control) were inoculated into 5 ml LB broth containing 50 µg ml^{-1} kanamycin and

cultured on a shaker at 100 revolutions/min for 48 h at 28°C. This suspension (50 μl) was then subcultured once in 125 ml-flask containing 50 ml of LB liquid medium and cultured overnight at the above conditions. Bacterial cells of an overnight culture were pelleted by centrifugation at 8,000 rpm at 25°C for 10 min and resuspended with MB liquid medium supplemented with 2 μg ml^{-1} BAP until final $absorbance_{595}$ equals 1.0. The equal volume of the same medium was added to prepare the bacterial suspension concentration of an $absorbance_{595}$ as 0.5 and used for inoculation.

1.2 Plant transformation and selection

A. Plant materials

Seeds of mungbean [*Vigna radiata* (L.) Wilczek] cv. Kamphaeng Saen 1 (KPS 1) and Suranaree 1 (SUT 1) were surface sterilized for 1 min. with 70 % ethanol and 15 min with 20 % Clorox plus 2 drops of Tween 20. Treated seeds were rinsed 3 times with sterile distilled water and germinated on MS (Murashige and Skoog, 1962) 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 lux).

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

1.2.1.1 Inoculation and hygromycin tolerance of mungbean hairy roots

Cotyledon explants were prepared from the 2-, 5- and 7-day-old mungbean cv. KPS 1 seedlings by making a horizontal slice through the hypocotyl

region, approximately 3-5 mm below the cotyledon. Subsequently, a lengthwise slice was made between the cotyledons and the embryonic axis was removed. This manipulation generated 2 cotyledonary node explants. A 30-ml overnight liquid culture of A. *rhizogenes* K599 carrying binary vector pCAMBIA 1301-choA was grown on LB medium supplemented with 50 μg ml^{-1} kanamycin. A culture of A. *rhizogenes* K599 lacking a binary vector (a control) was grown in the same medium without antibiotics in the same conditions. Each of the treated cotyledons was inoculated by uniform 4-6 vertical and horizontal slits on the abaxial surface by using a No. 11 scalpel blade previously dipped into the bacterial culture strain being tested.

Inoculated cotyledons were cultured abaxial side up on filter paper immersed in sterile distilled water and incubated at 24°C (experiment done in USA) and 25±3°C (experiment done in Thailand) in the dark. Three days after inoculation, cotyledons were transferred and cultured abaxial side up on MB medium [MS basal medium with Gamborg'S (1968) B_5 vitamins] containing 500 μg ml^{-1} timentin (TICAR: ticarcillin disodium, SmithKine Beecham, USA) with or without 25 μg ml^{-1} hygromycin and cultured at 28°C (experoment done in USA) and 25±3°C (experiment done in Thailand) under 16 h light. Timentin was added to inhibit the growth of *Agrobacterium* and hygromycin was added to test hygromycin sensitivity of the inoculated mungbean cotyledons.

Induced roots, 2-cm-long root tips, excised randomly from each treated cotyledon were assumed as one line of transformed root. Two or three induced roots per cotyledon were excised for propagation on a medium supplemented with or without 10, 25 and 40 μg ml^{-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 subculture for 2 times, histochemical GUS assay from each line was determined and plantlet regeneration was attempted.

1.2.1.2 Plant regeneration from hairy roots

Three-week-old hairy root, 3-cm long root tips were excised and transferred to fresh MB medium with and without growth regulator. To assess the influence of growth regulator, 0.1 and 0.5 μg ml^{-1} thidiazuron (TDZ), 0.5 μg ml^{-1} 6-benzylaminopurine (BAP), 1.0 μg ml^{-1} kinetin (KN) or indole-3-acetic acid (IAA) or α naphtalene acetic acid (NAA) were supplied in combination in the MB basal medium and cultured at 28°C under 16 h light.

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

1.2.2.1 The effect of seedling age on cotyledonary node transformation.

The cotyledonary nodes with proximal half of cotyledon attached of one-, two-, three-, and four-day old seedlings of mungbean cv. KPS 1 were excised followed by inoculation. For inoculation, the treated cotyledons were wounded at the cotyledonary node before transfer to 125-ml flask containing an overnight culture of bacterial suspension and placed in the laminar flow for 30 min. This inoculation was identified as a soybean method. Then, the inoculated cotyledonary node explants were transferred to co-culture on MB medium supplemented with 2 μg ml^{-1} BAP in the dark at 24°C for 3 days. After co-cultivation, the explants were transferred to culture on MB medium supplemented with 2 μg ml^{-1} BAP, with or without 25 μg ml^{-1}

hygromycin and 500 μg ml^{-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.

1.2.2.2 The effect of pre-incubation on cotyledonary node transformation.

Cotyledon explants from one-day-old seedling of mungbean cv. KPS 1 were pre-incubated on MB medium containing 2 μg ml^{-1} BAP for 1 and 2 days. Whereas cotyledon explants from two-day-old seedling were pre-incubated in that medium for 4 and 7 days at 28°C with 16 h photoperiod before inoculation. For inoculation, the treated cotyledons were wounded by 8-12 slits at the cotyledonary node while submerging in an overnight culture of bacterial suspension strain being tested. The co-cultivation, selection of transformed shoot and GUS assay were conducted as described in 1.2.2.1.

1.2.2.3 The effect of cultivars, co-culture conditions and culture conditions on cotyledonary node transformation.

Cotyledonary node explants of mungbean cv. KPS 1 and SUT 1 were germinated for 3 or 4 days and pre-incubated on MB medium containing 2 μg ml^{-1} BAP for 3, 5 and 7 days at 25±3°C with 16 h photoperiod before inoculation. The treated explants were inoculated by using hairy root method. The cocultivation and selection of transformed shoot was performed at 25±3°C. The treated explants were subcultured on that medium every 3 weeks. After two subcultures, the surviving cotyledons were determined for GUS assay.

1.2.2.4 The effect of acetosyringone and BAP concentrations on cotydonary node transformation.

Cotyledonary node explants of mungbean cv. KPS 1 and SUT 1 were germinated for 3 days and pre-incubated on MB medium containing 2 μg ml^{-1} BAP for 5 days at 25±3°C followed by inoculation by using hairy root method. The inoculated explants were cocultivated on MB medium containing 2, 5 and 10 μg ml^{-1} BAP with or without 100 μM acetosyringone. The selection and subculture was done as described in 1.2.2.3.

1.2.2.5 The effect of explants and wounding technique on mungbean transformation.

Hypocotyl and young leaves of 3-day-old seedling of cv. KPS 1 and SUT 1 were wounded by 2 methods before co-cultivation. For the first method, hypocotyl and young leaves were cut into 0.3 cm and 0.3 x 0.3 cm, respectively, in an overnight-grown inoculum of *Agrobacterium* suspension followed by 3-4 triangle excises. The second method was conducted by transferring 20 pieces of 0.3 cm hypocotyl and 0.3 x 0.3 cm young leaves into 5 ml of half MB medium containing 0.5 μg ml^{-1} KN (1/2 MB + 0.5 KN) in 125-ml flask and subsequently wounded by using sonicator (CAVITATOR ultrasonic cleaner, Metter Electronices Corp. Model : ME 4.6, serial 128M17438, Input watts 85) for 1, 3, 5, 7 and 10 min. The treated explants were then inoculated with *Agrobacterium* suspension for 30 min in the dark at 25±3°C. The inoculated explants from both methods were co-cultured on 1/2 MB plus 0.5 KN in the dark for 3 days at 25±3°C. Co-cultured hypocotyl and leaves, 36 and 9

explants per petridish, respectively, were cultured on the same medium supplemented with 500 μg ml^{-1} timentin for callus induction. These explants were subcultured at 2-week intervals.

B. Histochemical β–glucuronidase (GUS) expression assay

β–glucuronidase (GUS) activity was measured according to Cho *et al*. (2000) with some modifications. Tissues were transferred to a sterilized plastic petri dish containing the GUS assay buffer. The 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 prior to visual observation.

2 Cloning and expression of cholesterol oxidase gene (*choA*) in *E. coli* M15 (pREP4)

2.1 Construction of recombinant plasmids

A. Bacterial strains and plasmids

The *Escherichia coli* (*E. coli*) JM109 carrying the plasmid pCO117 (called strain JM109 pCO117) that has a cholesterol oxidase gene from *Streptomyces* sp. SA-COO was provided by Dr. Hyeon-Je Cho, University of Illinois, USA. The *E. coli* strain DH5α and pBluescript was used as a host for a cloning vector. The M15 carrying the plasmid pREP4 [M15 (pREP4)] was used as a host for an expression

vector (QIAGEN, USA). The plasmid pREP4 confers the kanamycin resistance and constitutively expresses the *lac* repressor protein encoded by the *lac*I gene. The QIAexpress vectors pQE 30, pQE 31 and pQE 32, the high-level expression of 6xHis-tagged proteins at the N-terminus were purchased from QIAGEN Inc, USA. The vectors pQE 30, pQE 31 and pQE 32 contained two *lac* operator/promoter and have the same ribosome binding site sequences but different at the beginning sequences behind the 6xHis-tagged fragment as shown in Figure 5. Thus, the DNA fragment must be inserted in the correct reading frame of this vector. So, the M15 will contain the repressor (pREP4) and the expression vector (pQE) with the inserted gene.

	*Bam*HI	*Hind*III
pQE 30	**_ _** GGATCC_ _	AAGCTTA
pQE 31	**AC** GGATCC_ _	AAGCTTA
pQE 32	**_ G** GGATCC_ _	AAGCTTA

Figure 5. Different sequences of the QIAexpress vector pQE 30, pQE 31 and pQE 32.

B. Media and culture conditions

Either Luria-Bertani (LB) or 2xYT medium was used as liquid and solid culture of the bacteria. The *E. coli* JM 109 pCO117 as well as the DH5α carrying the expression vector pQE 30, pQE 31 and pQE 32 and the pBluescript were grown in the presence of 100 μg ml^{-1} ampicillin (Sigma, USA). The M15 carrying the plasimd pREP4 was grown in the presence of 25 μg ml^{-1} kanamycin (Sigma, USA). All of the bacteria were grown either on solid or in liquid at 37°C. When cultured in liquid medium, the inoculum was shaken overnight at 180 rpm. The M15 carrying the plasmid pREP4 and pQE 30, pQE 31 or pQE 32 with or without the *choA* insertion was grown in the presence of both 25 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin.

C. DNA preparations

The DNA extraction from all of the *E. coli* used for plasmid construction was prepared by using QIAGEN plasmid Midi kit (QIAGEN, USA). The DNA extraction from all of the *E. coli* used for checking the ligation was prepared by using miniprep procedure according to Sambrook *et al* (2000) with slight modification by using P1, P2 and P3 buffer from QIAGEN plasmid Midi kit. Prior to subcloning, specific DNA fragments were fractionated by 0.8 % agarose (BIO-RAD, USA) gel electrophoresis, recovered and purified by Geneclean II kit (BIO 101 Inc, USA).

D. Preparation of competent cells

The preparation of the *E. coli* DH5α competent cells was described in 1C. The preparation of M15 (pREP4) competent cells was conducted according to The QIA*expressionist*™ (2000) (QIAGEN, USA). A single colony of bacterium M15

(pREP4) was overnight grown on LB broth medium containing 25 μg ml^{-1} kanamycin at 37°C before inoculation into 25 ml LB medium supplemented with the same antibiotic. After an A_{595} of the bacteril suspension was reached 0.5, the suspension was kept on ice for 5 min followed by centrifugation at 4,000 rpm, 4°C for 10 min. Then, the pellet was gently re-suspended with 7.5 ml of icy cold filter sterilized TFB1 and kept on ice for 90 min before centrifugation at the same condition. The pellet was gently re-suspended with 1 ml of icy cold filter sterilized TFB2 and was divided into 100 μl aliquots before storing at –80°C.

E. Ligation and transformation of *E. coli*

The ligation of a *choA* gene with pBluescript or pQE vectors was conducted according to Sambrook *et al* (2000). Then, the ligated DNA and the plasmid pCO117 were transformed to DH5α or M15 (pREP4) by heat-shock procedure according to Sambrook *et al* (2000) or The QIA*expressionist*™ (2000), respectively. The transformed cells were selected on a medium containing an appropriate antibiotic. The DH5α containing the plasmid pBluescript with a *choA* gene insertion was selected on LB medium supplemented with 100 μg ml^{-1} ampicillin. The M15 (pREP4) containing the plasmid pQE 30, 31 or 32 with a *choA* gene insertion was selected on LB medium supplemented with 25 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin. The surviving colonies on the medium were picked and cultured overnight in liquid medium supplemented with antibiotics for DNA extraction. The extracted DNA was cut with restriction enzymes *Bam*HI and *Hind*III and fractionated on 0.8 % agarose gel electrophoresis for the selection of a correct inserted *choA* gene size of 1.8 or 2.1 kb.

2.2 Selection of recombinant cells

The colonies grown on LB medium supplemented with 25 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin were further tested for *choA* activity according to Allain *et al* (1974). The colonies were streaked on LB medium supplemented with the same antibiotics and 1 mM IPTG (isopropyl-β-D-thigalactoside) and cultured overnight at 37°C. Colonies were transferred to Whatman paper No.1 (10 cm in diameter) and soaked in an enzyme cholesterol oxidase solution (see detail in 2.3.2.2) at 37°C for 15-30 min. The pink color confirmed a correct inframe sequence of the *choA* gene insertion.

2.3 Determination of intracellular and extracellular expression

2.3.1 Collection of extracellular and intracellular protein

The bacteria M15 (pREP4) containing the plasmid pCO117, as a control, and the plasmids pQE 30, pQE 31 and pQE 32 with the *choA* insertion were used in this study. Single colony each of these bacteria was inoculated in 5 ml LB medium supplemented with both 25 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin. The bacteria were grown overnight at 37°C with vigorous shaking. A 125 μl of the culture, which represents a starting $absorbance_{595}$ of approximately 0.005, was inoculated in 5 ml of fresh medium. The culture was grown until the $absorbance_{595}$ of 0.5-0.7 was reached. Before induction 1.5 ml of the sample was taken. An expression was induced by adding IPTG or no adding IPTG, as control, to the final concentration of 1mM. The cells were grown for 4 h before harvesting. The cells were kept on ice for 10 min before centrifugation at 12,000 rpm, 4°C for 10 min. The supernatants were collected

and used for an extracellular determination. These samples were kept at 4°C before use. The pellets were added with 0.1 M potassium phosphate buffer (K_2HPO_4 and KH_2PO_4, pH 7.0) and were sonicated (Vibra Cell^{TM}, Sonics and Materials, INC USA) at 50 amptitude for 6 times, 10 sec per each burst, to break the cells. The lysates were centrifuged at 12,000 rpm at 4°C for 5 min. Then, the supernatants were used as an intracellular determination. To avoid protein denaturation, all samples were kept on ice before use.

2.3.2 Determination of cholesterol oxidase activity

2.3.2.1 Determination of cholesterol oxidase protein

The cholesterol oxidase protein was first determined by Coomassie Brilliant Blue G-250 dye-binding assay using Bio-Rad protein assay dye reagent concentrate (BIO-RAD, USA) according to Bradford (1976). The protein concentration (μg) was corresponded on a standard curve of BSA (bovine serum albumin) concentration. The BSA curve was standardized with varying concentrations of BSA and measured the absorbance at 595 nm. Water was used as a blank check. The reaction mixture was composed of 790 μl water, 10 μl of protein sample and 200 μl of dye (concentrated). After adding a dye, the solution was mixed gently by inverting the tube. The measurement can be done between 10 and 30 min after adding of a dye.

2.3.2.2 Assay of cholesterol oxidase activity

The assay of cholesterol oxidase activity was performed by two methods. The first method was according to Allain *et al*. (1974) and the second method according to Masurekar and Goodhue (1978).

The principle of cholesterol oxidase activity by Allain *et al* (1974) was defined as the amount of enzyme producing 1 μ mole of H_2O_2 per minute at pH 7.0 and 37°C and 1 mol of H_2O_2 produced 0.5 ml of quinonemine dye. The sequence of reactions is shown below.

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} H_2O_2 + \text{4-cholesten-3-one}$$

$$H_2O_2 + \text{4-aminoantipyrine} + \text{phenol} \xrightarrow{\text{peroxidase}} 4H_2O + \text{quinonemine dye}$$

This method required enzyme solution that consisted of 0.1 mM potassium phosphate buffer, cholesterol solution, 1.76 % (w/v) 4-aminoantipyrine solution (Sigma, USA), 6 % (v/v) phenol solution and 1.5 mg/ml horseradish peroxidase (Sigma, USA). The solution was incubated at 37°C for 15 min before taking 3 ml into a new tube and added 50 μl of the purified cholesterol oxidase protein. The solution was mixed by vortexing for 15 sec before incubation at 37°C for 15 to 30 min. The decomposition rate of cholesterol oxidase was determined by measuring the rate of quinonemine developed (pink color) at absorbance$_{500}$ nm.

Enzyme solution was used as a blank. The enzyme cholesterol oxidase activity was calculated by using the formula below.

$$\text{Units/ml enzyme} = \frac{(\Delta A_{500}\ \text{nm/min test} - \Delta A_{500}\ \text{nm/min control}) \times 3.05}{13.8 \times 0.5 \times 1.0 \times 0.05 \times 15}$$

3.05 = volume in milliliters of assay

13.8 = millimolar extinction coefficient of quinonemine dye (oxidized) at A_{500} nm

0.5 = milliliters of quinonemine dye produced from the oxidization of cholesterol per minute

1.0 = mole of H_2O_2 produced from the oxidization of cholesterol per minute

0.05 = volume in milliliters of enzyme used

15 = incubation time (in min)

The principle of cholesterol oxidase activity according to Masurekar and Goodhue (1978) is defined by one unit of cholesterol oxidase that converts 1.0 μmole of cholesterol to 4-cholesten-3-one per minute at pH 7.5 and 25°C. The reduction of cholesterol in the presence of o-dianisidine and peroxidase produced the o-dianisidine (oxidized form) at A_{500} nm. The reaction is show below.

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} H_2O_2 + \text{4-cholesten-3-one}$$

$$H_2O_2 + \text{o-dianisidine (reduced)} \xrightarrow{\text{peroxidase}} 2H_2O + \text{o-dianisidine (oxidized)}$$

This method required enzyme solution that consisted of 50 mM potassium phosphate buffer, 0.5 % (w/v) cholesterol solution (100 mg cholesterol in 1 ml Triton X-100, 0.8 g cholic acid and adjust volume to 20 ml with distilled water), 1 % (w/v) o-dianisidine solution and 0.1 unit/ml horseradish peroxidase. The 3.0 ml reaction solution was mixed with 50 µl of the purified cholesterol oxidase protein and incubated at 25°C for 15-30 min before measurement the density of o-dianisidine at absorbance$_{500}$ nm. Enzyme solution was used as a blank. The enzyme cholesterol oxidase activity was calculated by using the formula below.

$$\text{Units/ml enzyme} = \frac{(\Delta A_{500}\ \text{nm/min test} - \Delta A_{500}\ \text{nm/min control}) \times (3.05)}{(7.5)\ (0.1) \times 15}$$

3.05 = volume in milliliters of assay

7.5 = millimolar extinction coefficient of o-dianisidine (oxidized) at A_{500} nm

0.1 = volume in milliliters of enzyme used

15 = incubation time (in min)

2.3.3 Assay of cholesterol oxidase specific activity

The enzyme specific activity (units per mg protein) was calculated by dividing enzyme activity with the protein amount as shown below.

$$\text{Units/ mg protein} = \frac{\text{enzyme activity (unit/ml)}}{\text{protein amount (mg/ml)}}$$

2.4 Time-course analysis of an expression

2.4.1 Time course analysis

Time course analysis was conducted to optimize the expression of a constructed clone, to check the presence of 6xHis-tagged protein at various times after induction and to optimize the induction period. In this study, one colony of the M15 bacterium carrying both plasmids pREP4 and pQE 30 with the *choA* insertion was overnight grown in 1.5 ml LB broth medium supplemented with 25 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin at 37°C. Fifteen ml of fresh LB medium was inoculated with 500 μl of an overnight-grown culture. The cells were cultured until an $absorbance_{595}$ of 0.5-0.7 was reached. Before induction, 1.5 ml of the culture was taken as a control. The expression was induced by adding the IPTG to the final concentration of 1 mM and continued culture for 5 h. One and a half ml of the sample was taken every 1 h. The cells were harvested and lysated as described above. The intracellular lysate protein was used for protein determination and enzyme specific activity determination. The Western blot was conducted to compare the cholesterol oxidase protein from the constructed clone with the commercial cholesterol oxidase protein from *Steptromyces* (Sigma, USA).

2.4.2 Western blot analysis

The choA protein, 10 μg before and after induction, was electrophoresed according to Laemmli (1970). The SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel eletrophoresis) was carried out on 7.5 % SDS Ready gel polyacrylamide eletrophoresis (Bio-Rad) using Bio-Rad Mini-Protein II Cell at 500

volts, 200 watts and 200 milliampares for 1.5 h in 1x SDS running buffer (prepared from 10x SDS running buffer that composed of 25 mM Tris-base, 1.92 M glycine and 1 % SDS). For visual detection of total protein (the second determination) gel was stained with Brilliant Blue G colloidal reagent (Sigma) that composed of 25 % isopropanol, 12.5 % acetic acid, 12.5 % methanol and 0.05 % Coomassie Brilliant Blue for 30 min, followed by quick destaining in buffer (25 % isopopanol, 12.5 % acetic acid and 12.5 % methanol) and then slowly destaining in a buffer (7.5 % acetic acid and 5 % methanol) for 1 h each. Cholesterol oxidase was detected by Western blot analysis (the third determination). Thus, the protein was transferred to nitrocellulose filters (Bio-Rad) by semi-dry electroblotting for 25 min at 25 V. Immunodetection was carried out using goat anti-cholesterol oxidase : HRP conjugated (Research Diagnostic, Inc) at 1 : 1,000 dilution in TBS (100 mM Tris-Cl, pH 7.4 and 0.9 % NaCl) and 1 % milk (Carnation). Alkaline phophatase-conjugated anti-goat IgG (Sigma) was used as the secondary antibody at 1 : 20,000 in TBS and 1 % milk. The reaction was detected using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad).

2.5 Purification of cholesterol oxidase protein

Purification of cholesterol oxidase was performed under native condition according to The QIA*expressionist*^{TM} (2000) with slight modification as follows. Inoculate single colony of M15 (pREP4) pQE 30-choA in 50 ml LB or 2xYT broth containing both 25 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin or 25 μg ml^{-1} carbenicillin (Sigma, USA) in a 125-ml flask. The cultures were grown overnight at 37°C with vigorous shaking. 500 ml of pre-warmed medium was inoculated with 20

ml of overnight-grown culture and grew at 37°C with vigorous shaking until an absorbance$_{595}$ was 0.5-0.7 (approximately 1.5 h). Before induction, 1.5 ml of the sample, as a control, was taken. The expression was induced by adding IPTG to the final concentration of 1 mM and phenylmethylsulfonylfluoride (PMSF, a serine protease inhibitor to prevent protein digestion) was added to the final concentration of 135 μM. After continuous culture for 4 h, the culture was kept on ice for 10 min. The cells were harvested at 3,000 rpm centrifugation, 4°C for 10 min. The supernatant was discarded and the pellet was re-suspended with 15 ml of cold lysis buffer A that composed of 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0. The solution was added with lysozyme and PMSF to the final concentration of 1 μg/μl and 135 μM, respectively. After mixed gently, the solution was incubated on ice for 30 min. The cells were lysated by using the sonicator 6 times, 10 sec per each burst. During this step, the cells must be kept on ice. The lysated cells were centrifuged at 10,000 rpm, 4°C for 30 min. The supernatant was mixed with 2 ml of 50% Ni-NTA (nickel-nitrilotriacetic acid) agarose resin at 4°C for 1 h. The mixer was slowly poured into a column (1.0 cm in diameter and 8.0 cm in length) and let it flow down by gravity. The protein binding with Ni-NTA was washed with cold washing buffer B that composed of 50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole, pH 8.0, for 5-10 column volume. The protein was eluted with cold elution buffer C that composed of 50 mM NaH_2PO_4, 300 mM NaCl and 250 mM imidazole, pH 8.0. The protein was collected by using 1.5 ml microfuge tube, 0.5 ml per each sample, until the A_{500} was constant. Each sample was added with 0.2 % BSA for protein stabilizer. The samples were kept at 4°C before use.

2.6 Factors affecting expression of recombinant plasmid pQE 30-choA

The inoculum age, medium, proteinase inhibitor, temperature, density of cells, IPTG concentration and cholesterol oxidase activity assay methods were studied. A single colony of M15 (pREP4) pQE30-choA was inoculated in LB medium supplemented with 25 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin at 37°C. The bacteria that grew overnight before inoculation in fresh LB medium was identified as an overnight-grown inoculum, whereas the bacteria that grew for 4 h before inoculation in fresh medium was identified as fresh inoculum. An overnight-grown inoculum and fresh inoculum were used in the study of age of inoculum. For studying the effect of the medium or temperature on expression of fusion protein, these bacteria were grown in either LB medium or 2xYT medium and at 30°C or 37°C. An overnight-grown bacteria at 0.25, 1, 2 and 4 ml were inoculated in 50 ml of fresh LB medium for study the density of inoculum on expression of the bacteria. For the study on the effect of proteinase inhibitor, the medium was added with or without 135 μM PMSF after A_{595} of 0.7 of the bacteria was reached. Similarly, the IPTG at 0.1, 0.5, 1.0, 1.5, 2.0 and 2.5mM was added at the same time to study the effect of IPTG concentration on expression of fusion protein. In addition, for the study of cholesterol oxidase activity assay methods the intracellularly crude cell lysates were used. The assay was preformed by using Allain *et al* (1974) method and Masurekar and Goodhue (1978) method. By using Allain *et al* (1974) method, the assay was conducted at 22°C and 37°C.

CHAPTER IV

RESULTS AND DISCUSSION

1. Mungbean transformation

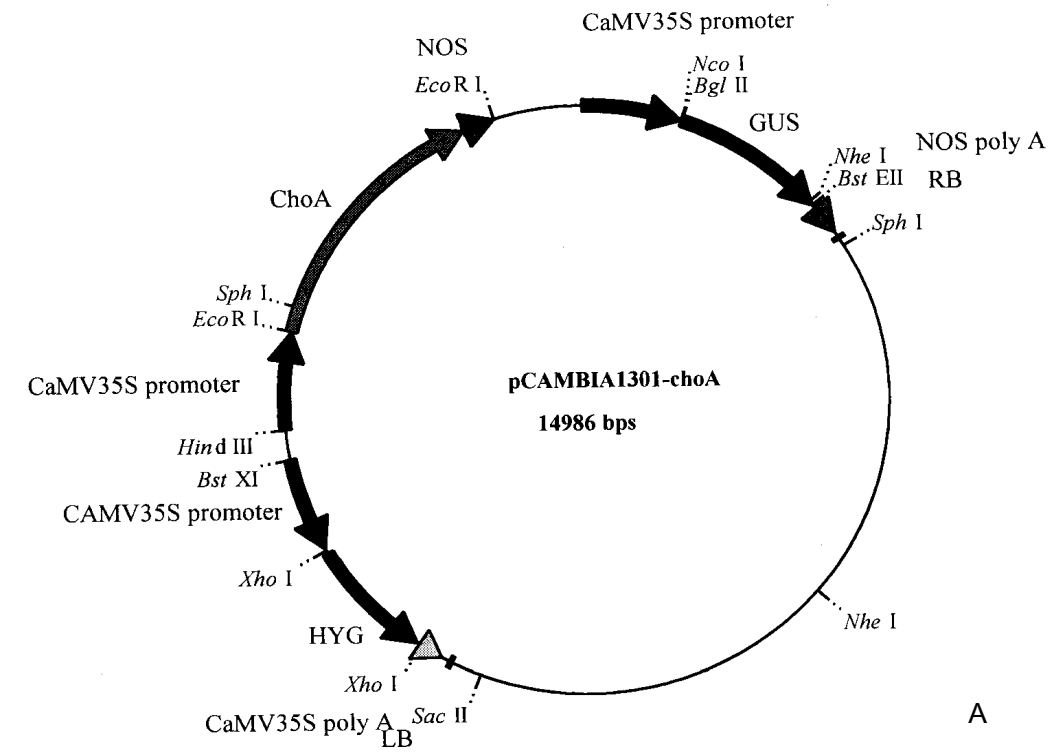
1.1 Plasmid construction for transformation

1.1.1 Plasmid construction for *Agrobacterium* transformation

The plasmid pBC4 extracted from *E. coli* DH5α was digested with *Hin*dIII (Gibco) followed by partial digestion with *Eco*RI to release a 2.8-kb fragment that contained the *choA* gene. The vectors pCAMBIA 1301 and pCAMBIA 3301 were digested with *Hin*dIII and *Eco*RI at the multiple cloning sites to release a linear fragment size 11.786 and 11.256 kb, respectively. Then, the *choA* fragment was inserted into *Hin*dIII and *Eco*RI sites of binary vector pCAMBIA 1301 and pCAMBIA 3301 by using T4 DNA ligase (BioLab, USA) at 16°C for 16 h. One μl of the ligated product was transformed to *E. coli* competent cell strain DH5α. After adding with 0.8 ml of LB medium and grew at 37°C for 1 h, 100 μl of bacterial agitation cells were plated on LB solidified medium containing 50 μg ml^{-1} kanamycin. After overnight incubation at 37°C, colonies were picked randomly and DNA extracted from selected clones was analyzed using restriction enzymes. The resultant vector pCAMBIA 1301 or pCAMBIA 3301 with *choA* insertion, size 14.986 and 14.516 kb, referred to as pCAMBIA 1301-choA and pCAMBIA 3301-choA, respectively, was transformed to

A. rhizogenes strain K599 and *A. tumefaciens* strain EHA 105 by electroporation. The engineeded *A. rhizogenes* strain K599 harboring binary vector pCAMBIA 1301-choA or pCAMBIA 3301-choA and *A. tumefaciens* strain EHA105 harboring binary vector pCAMBIA 1301-choA or pCAMBIA 3301-choA were shown in Figure 6. However, *A. rhizogenes* strain K599 and *A. tumefaciens* strain EHA105 with binary vector pCAMBIA 1301-choA were used for mungbean hairy root transformation and mungbean cotyledonary node transformation, respectiovely.

Since the strain of *A. tumefaciens* has an influence on transformation frequency, this experiment was designed to use *A. tumefaciens* strain EHA 105. In azuki bean, Yamada *et al*. (2001) found that *A. tumefaciens* strains AGL 1 and EHA 105 had higher transformation rate than that of LBA4404. AGL 1 and EHA 105 harbored a hypervirulent Ti plasmid and were developed to exhibit a broad host range and high transformation frequency (Hood *et al*., 1984; Jin *et al*., 1987; Hood *et al*., 1993). Such strains were reported to be efficient for transforming other legumes such as pea, peanut and lupin (Schroeder *et al*., 1993; Cheng *et al*., 1996; Bean *et al*., 1997; Pigeaire *et al*., 1997). However, Jaiwal *et al*. (2001) found that EHA 105 was not an optimal vector for regenerative tissues of mungbean.



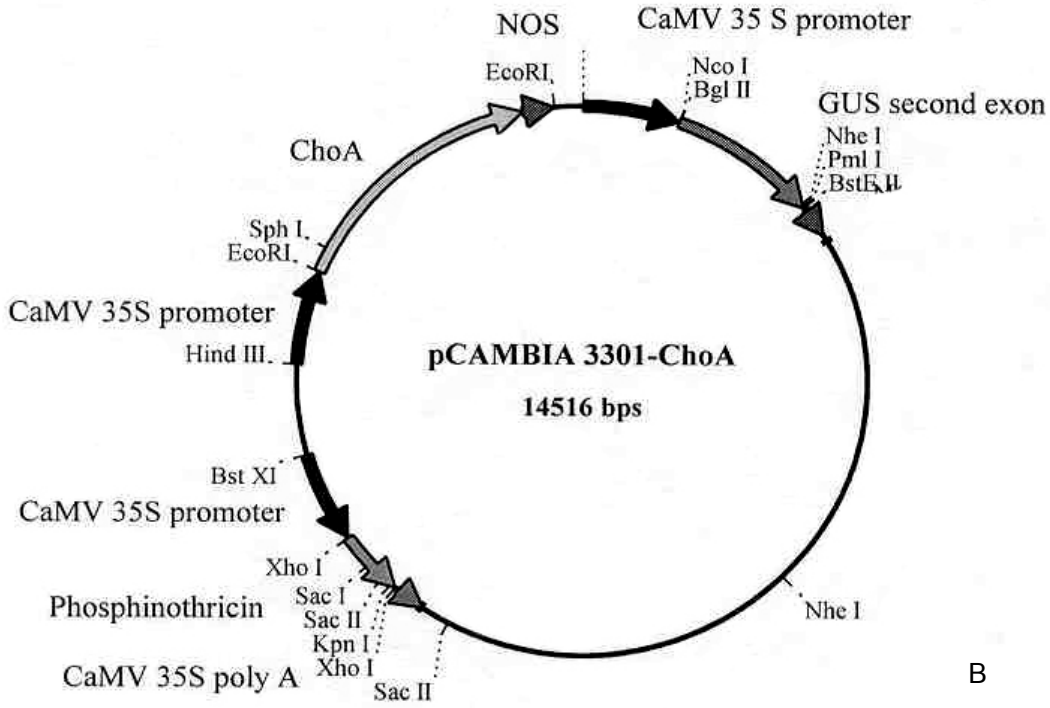


Figure 6. Schematic representation of (A) vector pCAMBIA 1301-choA and (B) binary vector pCAMBIA 3301-choA.

Also, this experiment used a binary vector pCAMBIA 1301 that had a *gus*A intron from castor catalase. An intron-interupted *gus* gene ensured that observed staining was due to only plant cell expression (Meurer *et al*., 1998). Zhang *et al*. (1997) reported that this assay system had at least two major advantages. It allowed *in situ* analysis and quantification of transformation event by directly observing GUS blue sectors in explant tissues. False reading caused by *Agrobacterium* contamination was eliminated due to the presence of an intron in the GUS coding region.

1.1.2 Plasmid construction for bombardment transformation

The 2.8 kb fragment of the *choA* gene was ligated with the 8.457 kb linear fragment of GUSpAPCH7 at the *Hin*dIII and *Eco*RI site. The ligated plasmid was transformed into DH5α competant cells by heat-shock procedure. The surviving colonies on the medium supplemented with 100 μg ml^{-1} ampicillin were picked randomly and DNA extracted from selected clones was analyzed using restriction enzymes. The GUSpAPCH7 with *choA* insertion, size 11.457 kb, was renamed as GUSpAPCH7-choA (Figure 7). Since the cotyledonary node explant was an unsuitable target, the bombardment technique was not performed in this experiment.

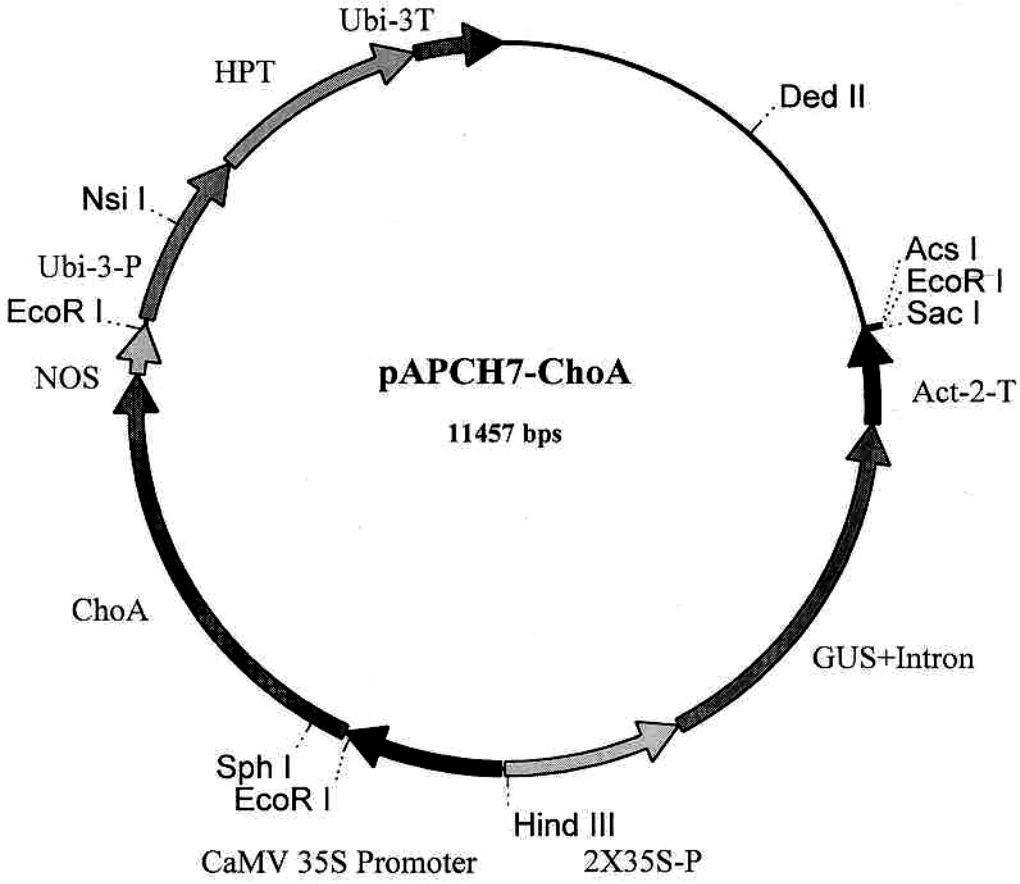


Figure 7. Schematic representation of GUSpAPCH7-choA.

1.2 Plant transformation and selection

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

1.2.1.1 Inoculation and hygromycin tolerance of mungbean hairy roots.

The inoculated cotyledons cultured on selective medium developed globular callus within 5-7 days at some parts of the abaxial side, as well as

adventitious roots directly developed without callus formation on the hypocotyl cut end (Figure 8A). Root primodia differentiated from callus tissue after culture on media without hygromycin in 10-14 days (Figure 8B).

The medium containing 25 μg ml^{-1} hygromycin severely inhibited growth of the cotyledons at different days after germination and on various treated cotyledons (Table 1, Figure 8C). Also, this concentration inhibited root developing from both the abaxial side and hypocotyl cut end. There were few small roots developed when hygromycin was presented. The ability of root induction was reduced progressively according to the age of cotyledon derived from seeds germinated in the medium without hygromycin. Two-day old cotyledon after germination showed higher ability to produce root (96.23 %) than the 5-and-7-day old cotyledons after germination (75.47 % and 42.86 %, respectively) as shown in Table 2. The observation was in agreement with those of Owens and Cress (1985) and Rech *et al*. (1989), who reported that the response of *Glycine max* seedlings to *Agrobacterium* strains carrying Ti plasmids or Ri plasmid was influenced by explant age. This could be due to the high efficiency of younger cells to grow and differentiate.

Moreover, these 2-day-old cotyledons when inoculated with K599 carrying the binary vector 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 14 days of infection (Figure 8D). On the contrary, the cotyledons wounded with a sterile scalpel (uninoculated control) showed 100 % producing of 1 tap root and 2-3 adventitious roots only from the hypocotyl cut end. Cotyledons inoculated with K599 lacking the binary vector

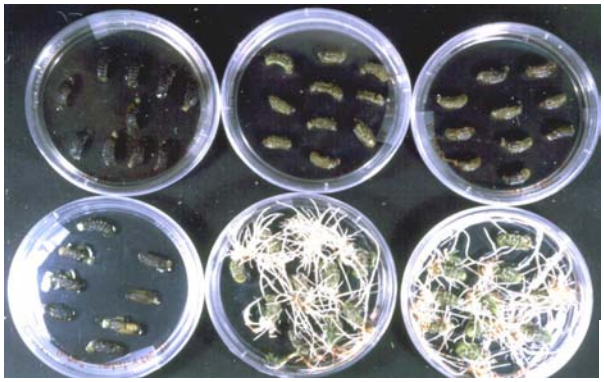
produced small amount of callus on abaxial side and roots on the hypocotyl cut end (Table 2).

Table 1. Effect of days after germination and medium containing 25 μg ml^{-1} hygromycin on root induction.

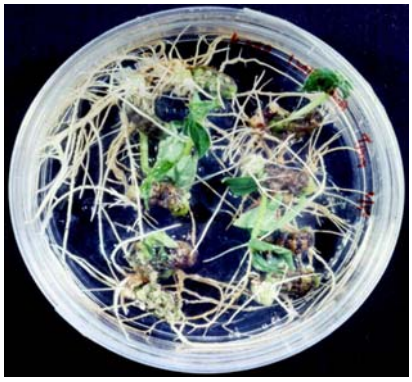
Treatment	Days after germination (d)	No. of inoculated cotyledons	No. of cotyledons with roots	% of root induction	No. of roots/ cotyledon
Uninoculated	2	13	5	38.46	1.02
	5	20	0	0	0
	7	20	0	0	0
K599	2	17	1	5.88	1.35
	5	20	1	5	1.13
	7	56	0	0	0
K599 pCAMBIA 1301-choA	2	20	0	0	0
	5	75	40	53.33	1.69
	7	93	1	1.08	1.06

A

B



C



D

Figure 8. Mungbean transformation with *A. rhizogenes*. (A) Callus induction after 10 days of culture. (B) Root primodia differentiated from callus tissue. (C) Hairy root 3 weeks after inoculation. Top-row plates show medium containing 25 μg ml^{-1} hygromycin and 500 μg ml^{-1} timentin, bottom row plates show medium without hygromycin (from left to right : control, K599 and K599 pCAMBIA1301-choA). (D) Induced roots, 0.5-7 cm, 3 weeks after culture on medium containing 500 μg ml^{-1} timentin.

Table 2. Effect of days after germination and the medium containing 500 μg ml^{-1} timentin on root induction.

Treatment	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

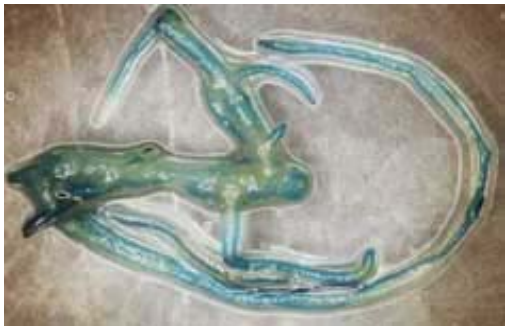
The induced roots were cut 2-3 cm long from the tip and transferred on to MB medium supplemented with 25 μg ml^{-1} hygromycin. A total of 75 out of 83 individual roots survived on the selective medium. Most of the roots exhibited rapid growth, formed lateral branches and grew not only down into the solid medium but also up into the air. This experiment resulted in three morphological types of transformed roots with respect to growth rate, branching, and degree of root geotropism, the same as previously reported by Cho *et al*. (1998; 2000). Type-1 : slow growth and thick roots; Type-2 : fast growing and highly branched roots and Type-3 : very fast growing and fine appearance. The possible explanation of such results may

be a differential expression of T-DNA genes present in the transformed roots, variable copy number of T-DNA inserted and positional integration effect of the T-DNA in the host genome. However, the morphological variations did not relate to the intensities of the hybridized signals probed with the *rolABC* genes (Cho *et al*. 2000). This observation was different from the results of Savka *et al*. (1990) who reported that there were no morphological differences among the transformed roots of various *G. max* genotypes.

From the total of 75 individual roots, a portion of each root was tested histochemically for GUS. Eleven roots were GUS positive (Figure 9A, B, C), which equal to 13.25 % of the tested roots. The intensity of the GUS activity varied from line to line. The different of GUS activity of the transformed roots was observed, it could be due to either the copy number of inserted genes or position effects arising from the integration of the gene (Fontana *et al*. 1993). As, Cho *et al*. (2000) suggested that the GUS activity assay could identify transformed tissues or patterns of gene expression, however, this test was not ideal for assay of transformant or the gene expression in living plant cells. Therefore, the study for identification of co-integration and expression of the *choA* gene by the assay of cholesterol oxidase activity and Western blot analysis must be conducted in plant tissue.

The GUS positive lines could survive on medium containing hygromycin selection medium up to 40 μg ml^{-1}. In this medium, however, the roots grew slowly with little branching and showed geotropism. In contrast, roots induced from uninoculated or inoculated with K599 lacking the binary vector stopped growing and died on the medium containing hygromycin. No GUS activity was observed in the tissue of uninoculated or inoculated with K599 lacking the binary vector (Figure 9D).

A



B

C

D

Figure 9. Transformed root showing GUS positive (A, B, C) and control (D).

The, 11 root clones were retained and made free from bacteria by two to three passages on a medium containing 500 μg ml^{-1} timentin. After propagating at 25±3°C, they were attempted to regenerate and from which were confirmed the cointegration of *choA* gene by assay of cholesterol oxidase activity, PCR, Southern blot, Northern blot and Western blot analysis. But these roots grew very slowly with brown color and died after 3 times of subculture. Thus, no molecular techniques were assessed on these lines.

The transformation methods also influenced the production of transformed roots. The protocol described by Cho *et al*. (2000) was used in this study due to its less laborious and less bacterial contamination when compared to the one reported by Savka *et al*. (1990). It took 6-8 weeks to establish *A. rhizogenes* decontaminated hairy roots, including 2-3 weeks for root induction and 4-5 weeks for bacterial decontamination. This new method also gave a high percentage of hairy root induction. In this study, the hairy root was induced at the percentage of 42.86-96.2 from treated cotyledons. Cho *et al*. (2000) reported the ability to induce hairy root at 54-95% from the wounded surface of the cotyledonary explants and all of the roots tested were found to be co-transformed when identified by cucumopine analysis, visual detection of GFP gene (green fluorescent protein) and polymerase chain reaction. However, this study found that only 13.25% of the tested lines were GUS positive. This could be that the induction was conducted on the medium containing only timentin, no hygromycin added as an inhibitor of untransformed roots. The previous report (Cho *et al*. 2000) suggested that its improvement may be due to the difference in culture methods and the cotyledonary node sites were responsive and highly efficient for transformation of hairy root.

Moreover, cotyledons of three-day-old seedling that were inoculated with *A. rhizogenes* at 25±3°C showed low efficiency of hairy root induction. The root emerged 2 or 3 weeks after cultured on induction medium without hygromycin. One to seven roots were observed per cotyledon. The induced root grew very slowly and one week after induction, the root tip dried up. Subsequently, individual root that was cut 2-3 cm-long and cultured on fresh medium did not promote the root growth. More than 80% of the roots died after the second passage of subculture. The survived roots (47 lines) were histocamically GUS assayed. The result showed that two lines were GUS positive. However, these lines could not survive on a medium containing 10 μg ml^{-1} hygromycin. Thus, no transgenic root was found from this experiment.

1.2.1.2 Plant regeneration from hairy root

Because of the difficulty of mungbean transformation by using cotyledonary nodes, the hairy root transformation and plantlet regeneration from hairy root was studied. The hairy roots of two weeks old after culturing on MB medium containing 0.5 μg ml^{-1} TDZ and 0.5 μg ml^{-1} BAP showed no differentiation. Root explants cultured on the medium containing 0.1 μg ml^{-1} TDZ and 1 μg ml^{-1} KN, or 0.1 μg ml^{-1} TDZ and 1 μg ml^{-1} IAA produced white friable callus, whereas the medium containing 0.1 μg ml^{-1} TDZ and 1 μg ml^{-1} NAA produced friable callus with green color. However, all of the calli grew very slowly on these medium. A small clump of callus excised from root explants did not grow well on fresh medium. The friable callus induced on the medium containing 0.1 μg ml^{-1} TDZ and 1 μg ml^{-1} KN, or 0.1 μg ml^{-1} TDZ6 and 1 μg ml^{-1} IAA died after the third subculture. Although, green callus

could survive on fresh medium containing 0.1 μg ml^{-1} TDZ and 1 μg ml^{-1} NAA, no differentiation was observed after subculturing for 6 times.

In many plant species, spontaneous shoots have been regenerated from the transformed roots produced following infection with *A. rhizogenes*. These species included some legumes such as *A. sinicus* (Cho *et al*., 1998), alfalfa (Golds *et al*., 1991; Spano *et al*., 1987), *Medicago arborea* (Damiani and Arcioni, 1991), *Lotus corniculatus* (Petit *et al*., 1987) and the wild perennial soybean species *G. canescens* (Rech *et al*., 1989). Many reports have indicated that addition of growth regulators, particularly cytokinins, to the culture medium can enhance shoot production from the hairy roots of some species such as horseradish (Saitou *et al*., 1992) and *G. argyrea* (Kumar *et al*., 1991). Kumar *et al*. (1991) reported the success of using plant growth regulator to regenerate shoots from hairy root of *G. argyrea* on B5 medium containing 0.2 μg ml^{-1} BAP and 0.005 μg ml^{-1} IBA. In mungbean, the transformed hairy root could not spontaneously regenerate. Therefore, growth regulators were added to the MB medium, but no plantlet from transgenic hairy root could be induced in this experiment.

1.2.2 Transformation with *A. tumefaciens* EHA 105 pCAMBIA 1301-choA

1.2.2.1 The effect of seedling age on cotyledonary node transformation.

Cotyledonary node of mungbean cv. KPS 1 was transformed by using soybean method. In four different seedling ages transformed with *Agrobacterium* EHA 105 pCAMBIA 1301-choA, only one cotyledon from 3-day-old seedlings

showed one blue sector of GUS activity at the base of the explant. Other seedling ages inoculated with the same bacteria showed GUS negative (Table 3). This result was similar to that of Jaiwal *et al*. (2001) who reported that cotyledonary nodes of two-day-old seedling of seven commercial cultivars of India showed higher transient transformation rates than those excised from four- or six-day-old seedlings. The younger explants might be more susceptible to *Agrobacterium* than the older ones. Thus, explant age had influence on transformation frequency.

As the protocol of cotyledonary node transformation offers one of the better methods for regeneration in soybean (Christou, 1990), there is still a major problem of chimerism for using this explant. Cotyledonary node is multicellular in origin, therefore, the shoot apical meristem presents problems similar to that found in soybean (Meurer *et al*., 1998; Yan *et al*., 2000) and chickpea (Fontana *et al*., 1993) transformation of cotyledonary node. In soybean, Meurer *et al*., (1998) reported the low efficiency of most common *A. tumefaciens*-mediated transformation system for soybean by using cotyledonary node.

Table 3. Effect of seedling ages on cotyledonary node transformation using soybean method.

Treatment	Days after germination (d)	No. of inoculated cotyledons	No. of cotyledons showing GUS positive
Control	1	30	0
	2	28	0
	3	30	0
	4	29	0
EHA 105	1	28	0
	2	29	0
	3	30	0
	4	30	0
EHA 105	1	60	0
pCAMBIA	2	65	0
1301-choA	3	63	1
	4	65	0

1.2.2.2 The effect of pre-incubation on cotyledonary node transformation

Cotyledon when pre-incubated on MB medium containing 2 μg ml^{-1} BAP before inoculation showed high transformation efficiency. The cotyledon derived from two-day-old seedlings that were pre-incubated for 4 days before inoculation showed the highest transformation frequency. Thirty-nine cotyledonary

nodes were inoculated, but only 32 cotyledonary nodes were assayed for GUS activity after culture for 2 weeks. The result showed that 10 out of 32 inoculated cotyledonary nodes had GUS activity with 1-3 blue sectors per explant (Table 4, Figure 10A, B, C and D). Additionally, one shoot that emerged from this GUS-positive cotyledon showed GUS activity on its shoot and leaf. (Figure 10E). The cotyledonary nodes of one-or two-day old seedlings that were pre-incubated for one, two and seven days before inoculation with the same bacteria and the cotyledonary nodes in other treatments showed no GUS activity. Although the pre-incubation for four and seven days promoted shoot formation more than the other treatments, no GUS positive was found in this experiment. In soybean, Santarem *et al*. (1998) reported no beneficial effect on transient transformation efficiency of the pre-incubation of cotyledons on induction medium. They also suggested that the effects of pre-incubation need to be re-evaluated for stable transformation work as the mitotic state of the target tissue may have different effects on transient and stable transformation.

Table 4. Effect of pre-incubation on cotyledon transformation using hairy root method.

Treatment	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	1	1	65	0
pCAMBIA		2	60	0
1301-choA	2	4	39	10
		7	37	0

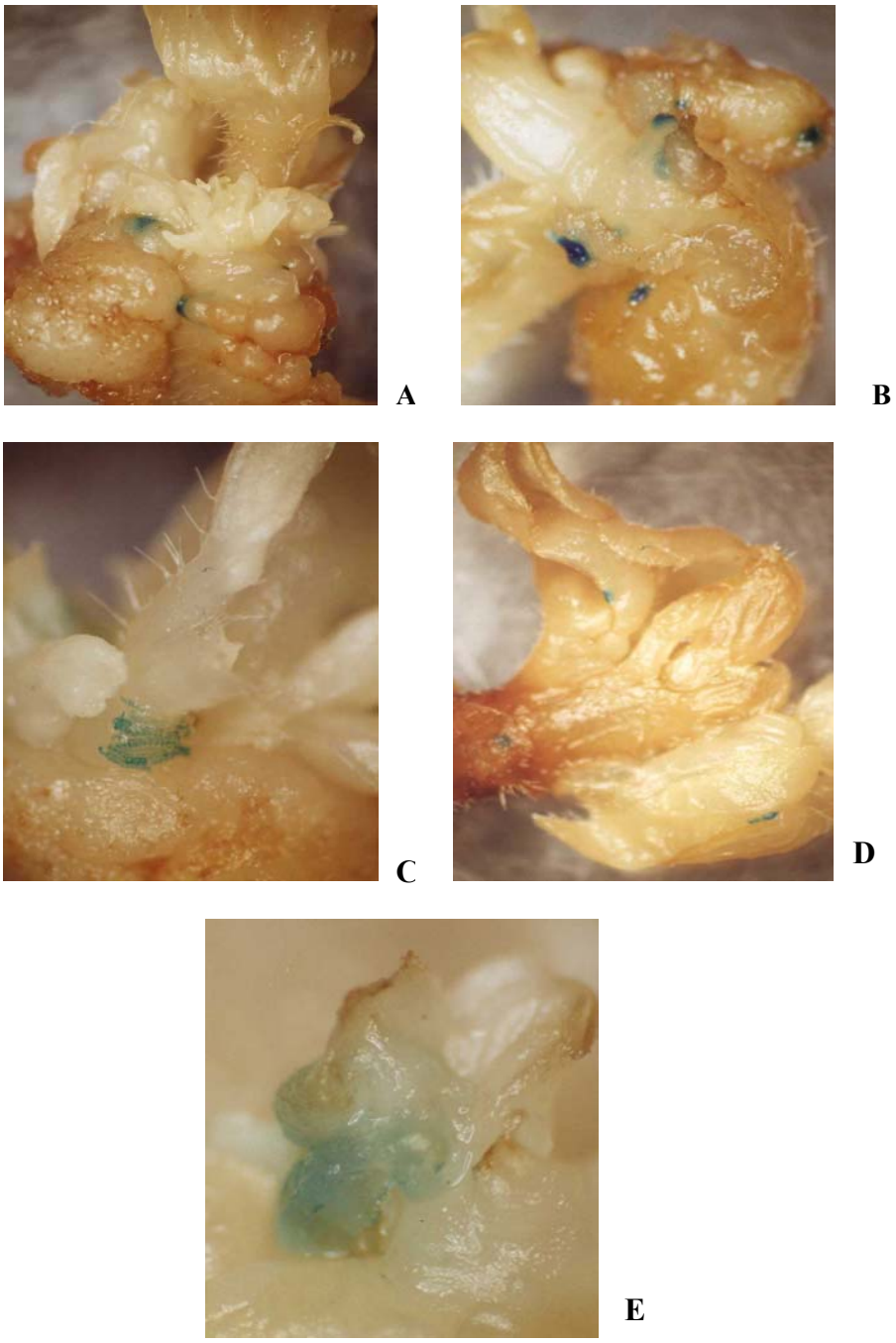


Figure 10. Cotyledonary node transformation with *A. tumefaciens*. A, B, C and D inoculated cotyledon with GUS positive. E shows shoot emerged from a GUS positive cotyledon.

1.2.2.3 The effect of cultivars, seed age, co-culture conditions and culture conditions on cotyledonary node transformation.

Since the mungbean seeds of cv. KPS1 and SUT 1 germinated at 25±3°C for 2 days were not suitable for cotyledonary node transformation, the best conditions were determined. At the mentioned temperature, the treated contyledonary nodes were assayed histochemically for GUS activity after two-week on selection medium. The results showed that all cotyledons were GUS negative (Table 5). The big shoots from both cultivars died after 4-6 subcultures. Small shoots did not elongate and finally died after 8 subcultures. However, cotyledonary nodes pre-incubated for 5 days before inoculation produced more small shoots than cotyledonary node pre-incubated for 3 and 7 days. The nodes of mungbean cv. KPS 1 and SUT 1 that were germinated, pre-incubated, inoculated, co-cultured and selected at 25±3°C could not produce shoot. Thus, this temperature was not suitable for mungbean cotyledonary node transformation. Although the mungbean seeds of cv. SUT 1 that were used in this experiment were freshly harvested, this condition did not enhance transformation efficiency. The same result was found in cv. KPS 1.

The result of this experiment was in contrast with Kudirka *et al*. (1986), who found that stem segments of soybean infected with *Agrobacterium*, co-cultured at 25°C enhanced transient expression, while higher temperature suppressed the transfer of T-DNA. Fullner and Nester (1996) concluded that temperature affected the efficiency of T-DNA transfer.

Length of co-cultivation period was reported to enhance transformation frequency. However, this experiment did not study the effect of duration of co-cultivation time. Because Jaiwal *et al*. (2001) reported that mungbean

cotyledons co-cultivated up to 3 days increased the transient transformation frequency. Further increase in co-cultivation time decreased the transformation frequency and resulted in bacterial over-growth. Therefore, in this experiment the co-cultivation was performed for only 3 days.

The experiment also showed that cultivars and seed age had an influence on mungbean transformation. Report of transformed mungbeans was obtained from cotyledonary nodes of seven commercial mungbeans grown in India, K-851, Pusa-105, ML-5, ML-323, ML-337, PDM-11 and PS-7 (Jaiwal *et al*., 2001). This experiment utilized the best Thai varieties, KPS 1 and SUT 1, but no transformation was obtained. However, it was observed that the younger seed age showed higher differentiation than the older seed age. This could be due to the damage and deterioration of the seed kept in storage that caused low transformation frequency.

Table 5. Effect of cultivar and seed age on shoot transformation at 25±3°C, 16 h light.

Treatment	Cultivars	Pre-incubation (days)	No. of inoculated cotyledon	No. of cotyledon showing GUS positive
Control	KPS 1	3	48	0
		5	35	0
		7	28	0
	SUT 1	5	29	0
		7	30	0
EHA 105	KPS 1	3	79	0
		5	37	0
		7	34	0
	SUT 1	5	40	0
		7	39	0
EHA 105 pCAMBIA 1301-choA	KPS 1	3	775	0
		5	555	0
		7	276	0
	SUT 1	5	548	0
		7	434	0

1.2.2.4 The effect of acetosyringone and BAP concentrations on cotyledonary node transformation.

The cotyledonary nodes that were co-cultured on MB medium containing 10 μg ml^{-1} BAP and 100 μM acetosyringone for 3 days produced more multiple shoots than that co-cultured on the medium containing 2 and 5 μg ml^{-1} BAP

and 100 μM acetosyringone. Seven to ten shoots per explant were produced on this medium as compared to 1-4 and 3-7 shoots per explant regenerated on the medium supplemented with 2 and 5 μml^{-1} BAP, respectively (Table 6). Additionally, shoots emerged from the medium containing 10 $\mu g\ ml^{-1}$ BAP and 100 μM acetosyringone grew quicker than that emerged from the latter media. However, half of the fast growing shoots from all experiments died after first subculture on selection medium. The cause of death was unknown. After four subcultures of big shoots on selection medium, these shoots showed stunted growth and finally died. This experiment indicated that acetosyringone did not enhance transformation of mungbean. Similar results were also reported in peanut by Mansur *et al*. (1993) and Cheng *et al*. (1996). In pea, De Kathen and Jacobsen (1990) found that acetosyringone decreased the transformation rate. However, the result of the latter study was in contrast with report of Godwin *et al*. (1991). Santerem *et al*. (1998) also found acetosyringone enhance transient expression of GUS in soybean. In addition, in azuki bean, addition of acetosyringone alone had no obvious effect on transient GUS expression but a high concentration of BAP up to 10 $\mu g\ ml^{-1}$ with 100 μM acetosyringone caused efficient gene transfer by *Agrobacterium*. However, it was observed that when 100 μM acetosyringone and 5 or 10 $\mu g\ ml^{-1}$ BAP were presented in co-cultivation medium, several big shoots were produced, but no GUS activity was found.

Table 6. Effect of acetosyringone (AS) and BAP concentrations on cotyledonary node transformation.

Cultivars	AS (μM)	BAP (μg/ml)	No. of inoculated cotyledons	No. of cotyledons producing shoots	No. of shoots per cotyledon
KPS 1	0	2	90	88	1-4
	100	2	93	91	1-4
	100	5	92	92	3-7
	100	10	95	95	7-10
SUT 1	0	2	97	95	1-4
	100	2	95	93	1-4
	100	5	92	92	3-7
	100	10	94	95	7-10

1.2.2.5 The effect of explants and wounding technique on mungbean transformation.

Since low transformation efficiency of cotyledonary node was found when the culture condition was performed at 25±3°C, the use of callus system to optimize transformation procedure was conducted in this experiment. Hypocotyl and leaf from two-day-old seedlings were used as explants for callus induction and ultrasonic apparatus was used for making wounds. The hypocotyl and leaf explants were ultrasonicated for 1, 3, 5, 7 and 10 min before inoculation with a bacterium suspension followed by co-culture in the dark for 3 days at 25±3°C. The result showed that none of the leaves wounded with either this methods produced calli. The leaves turned brown and died after being cultured on the medium for 3-4 weeks. A few friable calli were observed on the hypocotyl wounded with both methods after culture

on callus induction medium for 3 weeks. However, the percentage and number of calli were very low. Two weeks after first subculture, the calli turned brown and died, whereas the hypocotyl without calli died 3 weeks after culture. Thus, transgenic calli were not obtained from this experiment.

Joersbo and Biunsledt (1992) suggested that the intensity of the sonication treatment had to be carefully monitored to control microwounding and cell disruption. High-intensity ultrasound resulted in immediate cell lysis. In soybean, Santarem *et al*. (1998), obtained the highest GUS expression when immature cotyledons were sonicated for 2 seconds followed by co-cultivation for 3 days at 27°C. Cotyledons sonicated for 2 seconds had moderate tissue disruption, while the longer treatment resulted in more extensive damage. Therefore, unsuitable intensity of sonication treatment in this experiment might cause explant damage and revealed no further proliferation of the tissue.

In transformation work, various antibiotics have been employed to eliminate *Agrobacterium*. Currently, carbenicillin and cefotaxime are the most commonly used antibiotics for suppressing *Agrobacterium* (Nauerby *et al*., 1997). More recently, timentin has been made available and it showed a marked efficiency for eliminating *Agrobacerium* and also has stimulatory effects on organogenesis (Schroeder *et al*., 1993; Zimmerman, 1995). This experiment used timentin at the concentration of 500 μg ml^{-1} for four subcultures and gradually decreased to 400, 300, 100 and 50 μg ml^{-1} in each subculture. This antibiotics is a mixture of ticarcillin, a penicillin derivative, and clavulanic acid (Nauerby *et al*., 1997). Although an ideal antibiotic for inhibiting *Agrobacterium* sp. should be highly effective, inexpensive, without a negative effect on plant growth and regeneration and stable in culture

(Cheng *et al*. 1998), timentin was observed to cause an increase in morphogenesis of *in vitro* cotyledon explants of tomato (Costa *et al*., 2000; Ling *et al*. 1998). Timentin-supplemented media, 300 μg ml^{-1}, tended to give higher number of shoots per explant as suggested by Frary (1995) and Frary and Earle (1996). However, this experiment did not study the effect of timentin on shoot induction

2. Cloning and expression of cholesterol oxidase gene (choA) in *E. coli* M15 (pREP4)

2.1. Construction of recombinant plasmids

The plasmid pCO117 extracted from the *E. coli* JM 109 was cut with restriction enzymes, *Eco*RI (Gibco, USA) and *Hin*dIII (Gibco, USA) or *Sph*I (Gibco, USA) and *Hin*dIII to release a 2.0-kb and a 1.6-kb DNA fragment coding for cholesterol oxidase gene, *choA* or *choa*, with and without first ATG and NH_2-terminal sequence favorable in *E. coli* and *Stretomyces*, respectively. The 1.6-kb DNA fragment was directly ligated to *Sph*I and *Hin*dIII site of linear vector pQE 30 yielding an inframe reading recombinant DNA pQE30-choa. Since, the *Eco*RI sequence was not shown in the multiple cloning site of the expression vectors, the 2.0-kb DNA fragment was subcloned into the *Eco*RI and *Hin*dIII site of vector pBluescript and was renamed pBluescript-choA. The pBluescript-choA was cut with *Bam*HI (Gibco, USA) and *Hin*dIII to release the choA gene before ligating into *Bam*HI and *Hin*dIII site of the expression vectors pQE 30, pQE 31 and pQE 32. The recombinant plasmids were renamed pQE 30-choA, pQE 31-choA and pQE 32-choA. The ligation of vector pQE

30 with a *choA* gene insertion resulted in an inframe reading sequence with additional 8 amino acids as shown in Figure 11.

The recombinant plasmids pQE 30-choa, pQE 30-choA, pQE 31-choA and pQE 32-choA were transformed into *E. coli* DH5α for maintaining before being transformed into *E. coli* M15 carrying plasmid pREP4. For checking of the insertion, DNA was extracted from these transformant cells and cut with *Bam*HI and *Hin*dIII before being fractionated on 0.8 % agarose gel. The gel showed *choA* gene inserted with vectors pQE 31 and pQE 32 (Figure 12A). Due to the inframe reading sequence, the expression of *choA* or *choa* gene in *E. coli* DH5α caused toxicity to the cells. Thus, the inserted *choA* or *choa* gene was not found on the gel (figure 12A and B). For checking of these events, *choA* gene and 0.8-kb DNA fragment coding for 35S promoter was ligated with vector pQE 30 and pUC 19 before transformation into DH5α and a host cell, M15 (pREP4). The results showed that the constructed plasmids pUC19-choA, pUC19-35S and pQE30-35S extracted from DH5α and pQE 30-choA and pQE 30-35S extracted from M15(pREP4) had the inserted gene shown on the gel (Table 7, Figure 13A-F). This event could be described as the pQE vector having *lac* operator sequences and plasmid pREP4 contains $lacI^q$ gene. So, the expression of recombinant protein in M15 encoded by pQE vector was rapidly induced by the addition of IPTG which bound to the *lac* repressor proteins produced from $lacI^q$ gene of plasmid pREP4 and inactivates it. With this system, the expression of plasmid pQE30-choA or pQE30-choa in *E. coli* M15 (pREP4) showed no toxicity to the cells. On the other hand, *E. coli* DH5α had no system to control the expression of cholesterol oxidase protein, the overproduction of this protein resulted from

(A)

*Eco*RI ↓ ↓ *Hin*dIII

GAATTC **ATG** ACT GCA CAA CAG CAT CTG TCC CGT CGT CGT A….. AAGC TTA

M T A Q Q H L S R R R

(B)

*Bam*HI *Eco*RI ↓ ↓ *Hin*dIII

GAA CTA GTG GAT CCC CCG GGC TGC AGG AAT TCG ATA TCA AGC TTA TCG A

(C)

*Bam*HI ↓ *Eco*RI

GAA CTA GTG GAT CCC CCG GGC TGC AGG AAT TCA **TG**A CTG CAC AAC AGC

↓ *Hin*d III

ATC TGT CCC GTC GTC GTA TGC TGG GT….AAG CTT A

(D) *Bam*HI

pQE 30-choA GGA TCC CCC GGG CTG CAG GAA TTC **ATG** ACT GCA CAA CAG

*Hin*dIII

CAT CTG TCC CGT CGT CGT ATG CTG GGT…..AAG CTT A

*Bam*HI

pQE 31-choA ACG GAT CCC CCG GGC TGC AGG AAT TCA **TG**A CTG CAC AAC

*Hin*dIII

AGC ATC TGT CCC GTC GTC GTA TGC TGG GT…..AAG CTT A

*Bam*HI

pQE 32-choA GGG ATC CCC CGG GCT GCA GGA ATT CA**T G**AC TGC ACA ACA

*Hin*dIII

GCA TCT GTC CCG TCG TCG TAT GCT GGG T…..AAG CTT A

Figure 11. Construction of the expression vectors pQE 30, 31 and 32 with *choA* gene insertion. (A) Plasmid pCO117 cut with *Eco*RI and *Hin*dIII to release a *choA* gene. (B) Plasmid pBluescript II SK (+/-)cut with *Eco*RI and *Hin*dIII. (C) Plasmid pBluescript-choA cut with *Bam*HI and *Hin*dIII to release a *choA* gene. (D) Plasmids pQE 30, 31 and 32 ligated with *choA* gene at *Bam*HI and *Hin*dIII site.

transcription and translation of this plasmid in the bacterial cells was accumulated and finally made toxicity to the cells.

In this study, the recombinant plasmids pQE 30-choA, pQE 30-choa, pQE31-choA, pQE 32-choA and pCO 117 were transformed into M15 (pREP4) and used for further study.

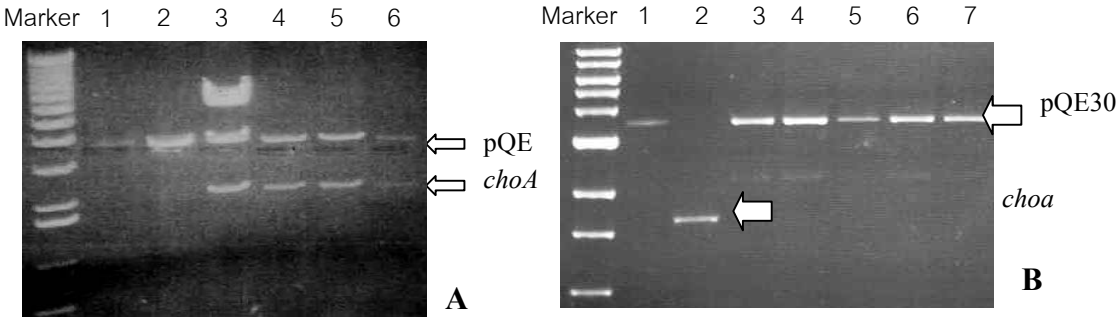


Figure 12. Ligation of *choA* with expression vectors. (A) Top band of lane 1, 2 = vector pQE 30, lane 3, 4 = vector pQE 31 and lane 5, 6 = pQE 32, bottom band of lane 3, 4, 5, 6 = *choA*. (B) The ligation of *choa* with vector pQE 30. Top band of lane 1 and 3-7 = pQE 30, lane 2 = *choa*.

Table 7. Insertion of *choA*, *choa* or 35S in various vectors and hosts.

Plasmid	Host cells and antibiotics	Insertion verification
pQE 30 - 35S	DH5α, Amp 100	Yes
pQE 30 - choA	DH5α, Amp 100	**No**
pQE 30 - choa	DH5α, Amp 100	**No**
pUC 19 - 35S	DH5α, Amp 100	Yes
pUC 19 - choA	DH5α, Amp 100	Yes
pQE 30 - 35S	M15 (pREP4), Amp 100 Kan 50	Yes
pQE30 - choa	M15 (pREP4), Amp 100 Kan 50	Yes
pQE 30 - choA	M15 (pREP4), Amp 100 Kan 50	Yes

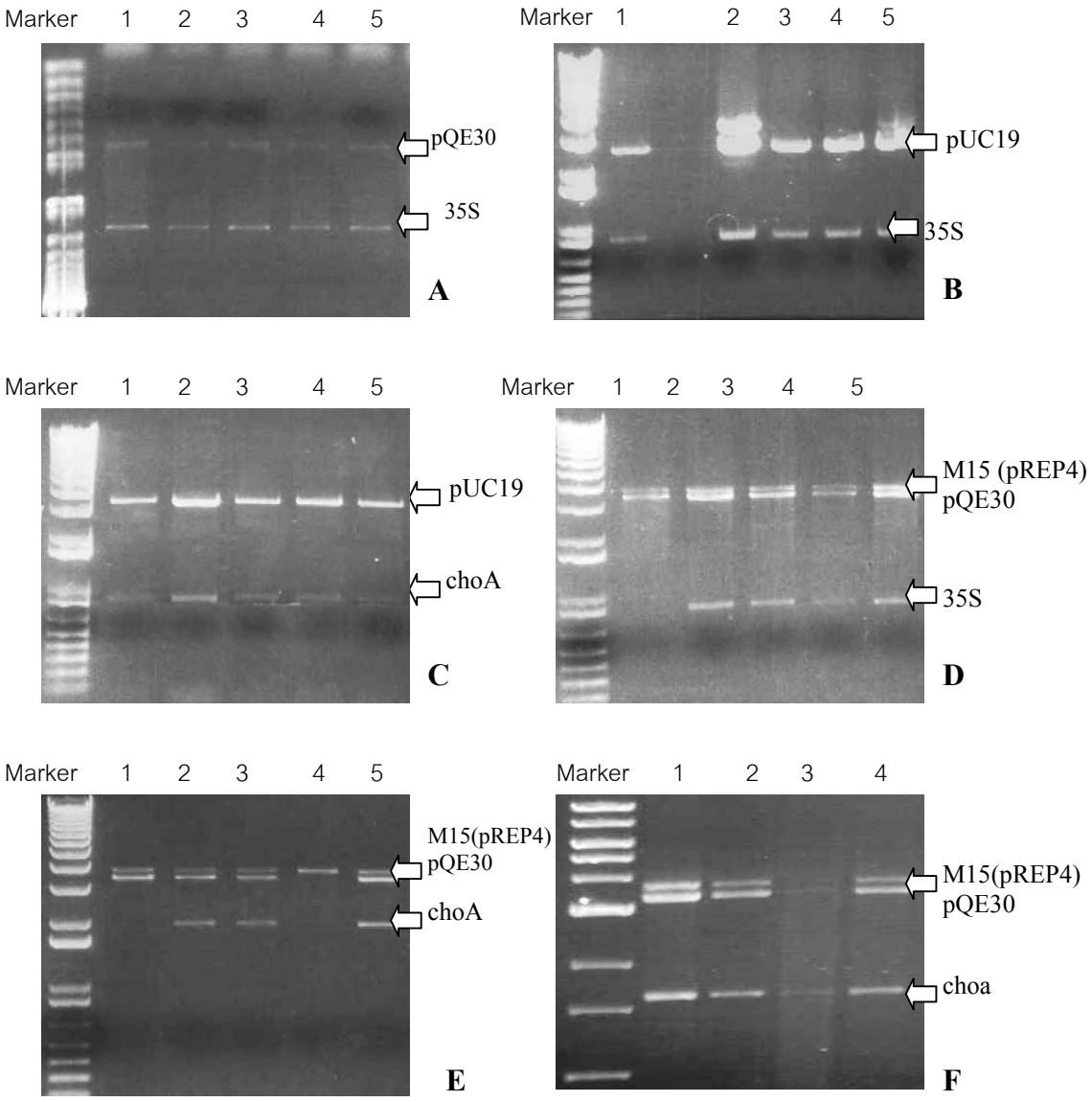


Figure 13. Insertion of *choA*, *choa* or 35S in various vectors and hosts. Top band of 1-5 A = pQE 30, 1-5 B, C = pUC19, 1-5 D, E, F = M15 (pREP4) pQE 30. Bottom band of 1-5 A, B, D = 35S promoter, 1-5 C, E = *choA*, 1-4 F = *choa*.

2.2 Selection of recombinant cells

Recombinant plasmids were first selected for an expression of cholesterol oxidase enzyme by detection of pink color that occurred in the presence of enzyme solution. Colonies were transferred to Whatmam paper and soaked in the enzyme cholesterol oxidase solution at 37°C for 30 min. The M15 (pREP4) containing plasmid pCO117, a positive control, and pQE 30-choA showed pink color, whereas the recombinant plasmid M15 (pREP4) containing pQE 31-choA and pQE 32-choA yielded fair color. Since, the *choa* gene contained no promoter, the ribosome binding site and the 5′ region of the original *choA* gene, no color appeared from this clone. Thus, this plasmid was not used for further study. Also, the negative control clones pQE 30, pQE 31 and pQE 32 contained no inserted *choA* gene, no color could be detected.

2.3 Determination of intracellular and extracellular expression.

The extracellular and intracellular expression of *E. coli* M15 (pREP4) carrying plasmid pCO 117, pQE 30, pQE 31, pQE 32, pQE 30-choA, pQE 31-choA and pQE 32-choA were determined. The protein concentration (mg ml^{-1}) and enzyme cholesterol oxidase activity (U ml^{-1}) were calculated for enzyme specific activity (U mg^{-1} protein). The results showed that enzyme cholesterol oxidase expressed in M15 (pREP4) system was an intracellular protein. No cholesterol oxidase activity was detected in culture broth of this bacterium. The reason was that the *choA* gene used in this study was modified by Nomura *et al*. (1995) to contain signal peptide sequences that favored both *E. coli* and *Streptomyces*. Thus, the *E. coli* failed to recognize the *Streptomyces* sp. cholesterol oxidase signal peptide sequences and could be able to

accumulate the enzyme in the cell. On the other hand, the negative clones (pQE 30, pQE 31 and pQE 32) contained no inserted gene. No enzyme activity could be detected in the cell or in the culture broth. In case of non-inframe reading sequences, pQE 31-choA and pQE 32-choA contained the frame-shift of *choA* gene, therefore, transcription and translation of this gene was changed and resulted in poor enzyme activity occurring in the cell.

After induction with 1 mM IPTG for 2 h, the positive control clone showed the highest yield of cholesterol oxidase enzyme activity (560 mU mg protein^{-1}). An inframe reading sequence, pQE 30-choA showed an activity of 263 mU mg protein^{-1}. An *E. coli* carrying non-inframe reading sequence or negative clone produced less cholesterol oxidase than those carrying an inframe reading sequence. The non-inframe reading sequencs, pQE 31-choA and pQE 32-choA showed the activity of 65 and 72 mU mg protein^{-1}, respectively, whereas the negative clones pQE 30, pQE 31 and pQE 32 showed the activity of 9, 10 and 9 mU mg protein^{-1}, respectively (Table 8).

Table 8. Determination of cholesterol oxidase gene expression in *E. coli* M15 (pREP4) carrying various plasmids[a].

Plasmids	IPTG	Enzyme activity (mU/ mg protein)	
		Extracellular	Intracellular
pCO117	–	ND[b]	137
	+	ND	560
pQE 30	–	ND	8
	+	ND	9
pQE30-choA	–	ND	317
	+	ND	263
pQE 31	–	ND	7
	+	ND	10
pQE31-choA	–	ND	7
	+	ND	65
pQE 32	–	ND	5
	+	ND	9
pQE32-choA	–	ND	9
	+	ND	72

[a]The cells were grown for 5.5 h in 5 ml LB medium with 25 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin and with (+) or without (–) IPTG.

[b]ND = Not detected

Although, the *choA* gene inserted in plasmid pQE 30-choA was cut from the plasmid pCO117, there were differences in intracellular enzyme production between *E. coli* M15 (pREP4) that carried plasmid pCO117 and pQE30-choA. This event was resulted from its promoter. In plasmid pCO117 the *tac* pormoter of pKK223-3 was located, whereas plasmid pQE30-choA contained two *lac* promoters. It seemed likely that the expression of pQE30-choA would be more efficient than the expression of pCO117, but the result obtained was not as such. Nomura *et al*. (1995) also reported that the efficiency of *lac* and *tac* promoters for the expression of the *choA* gene was significantly different between pCO103 (10 mU mg^{-1} protein) and pCO117 (1,464 mU mg^{-1} protein). Solaiman and Somkuti (1991) also found that the expression of extracellular cholesterol oxidase or *choA* gene under the control of *lac* Z promoter produced 7 mU mg $protein^{-1}$. Sampson and Chen (1998) found that the promoter and modified gene encoding the expression codons of *E. coli* enhanced expression of *B. sterolicum* cholesterol oxidase gene in *E. coli*. Additionally, different host cells had an effect on expression efficiency. Nevertheless, the recombinant plasmid pQE 30-choA produced more cholesterol oxidase before induction than after induction. This occurrence could be due to the high copy number of the expression vector.

2.4 Time course analysis of expression

Since, the negative clones and non-inframe reading sequence clones showed no cholesterol oxidase activity, time course analysis of these clones was not studied. The activity of cholesterol oxidase in *E. coli* M15 (pREP4) carrying plasmid pCO117 or pQE 30-choA was significantly higher than in M15 (pREP4) carrying plasmid pQE 30.

The time course of intracellular cholesterol oxidase production by M15 (pREP4) carrying both plasmids is shown in Table 9 and Figure 14. The maximum enzyme activity obtained from clone M15 (pREP4) carrying plasmid pCO117 (495 mU mg protein^{-1}) and the recombinant plasmid pQE 30-choA (351 mU mg protein^{-1}) was detected after induction for 2 and 4 h, respectively. After that period, the activity in both bacteria appeared to decrease slowly. Cholesterol oxidase obtained from M15 (pREP4) carrying plasmid pQE 30-choA after induction with 1 mM IPTG was 67.4 folds more than pre-induction. However, the enzyme detected from M15 (pREP4) that carried plasmid pQE 30-choA showed lower activity than did the control clone throughout the time course. The SDS-PAGE of crude cell lysate from M15 (pREP4) carrying plasmid pCO117 and pQE 30-choA showed the same molecular weight of 57 kDa after induction for 4 h (Figure 15). Since, no cholesterol oxidase protein was produced before induction, no distinct band was shown. On the other hand, the negative clone M15 (pREP4) pQE 30 showed no band of cholesterol oxidase protein from either before or after induction.

This study found that enzyme activity produced from clone M15 (pREP4) pQE 30-choA was not as high as expected. The QIAexpressionist handbook suggests that the inserted DNA sequences may encode elements that interfere with transcription or translation and internal starts should be eliminated by deleting any Shine-Dalgarno sequences in the coding region. Thus, the *choa* gene without first ATG, NH_2-terminal sequences and promoter was ligated with expression vector pQE 30 resulted in an inframe reading sequence. But no enzyme cholesterol oxidase activity was produced from that clone. The result confirmed that those sequences, single or in combination, affected the *E. coli* transcription and translation process. The result reported here was

in agreement with Nomura *et al* (1995). They suggested that the over-production of cholesterol oxidase of pCO117 in *E. coli* JM109 seem to have resulted from the alteration of either the Shine-Dalgarno sequences, the initiation codon, or the 5′ region of *choA* gene or from the combined effect of their alteration.

Table 9. Time course analysis of expression of M15 (pREP4) containing plasmid pCO117, pQE 30 and pQE 30-choA

Time after	Growth rate (A_{595})			Enzyme activity (mU/ mg protein)		
induction (h)	pCO117	pQE30	pQE30-choA	pCO117	pQE30	pQE30-choA
0	0.706±0.33	0.732±0.28	0.884±0.20	52	9	6
1	1.174±0.31	1.141±0.19	1.407±0.24	211	6	163
2	2.107±0.27	2.273±0.25	1.161±0.18	495	6	247
3	1.998±0.14	2.569±0.21	2.002±0.26	466	6	338
4	2.100±0.34	3.072±0.14	2.263±0.17	429	5	351
5	2.223±0.29	3.078±0.22	2.454±0.22	426	6	345

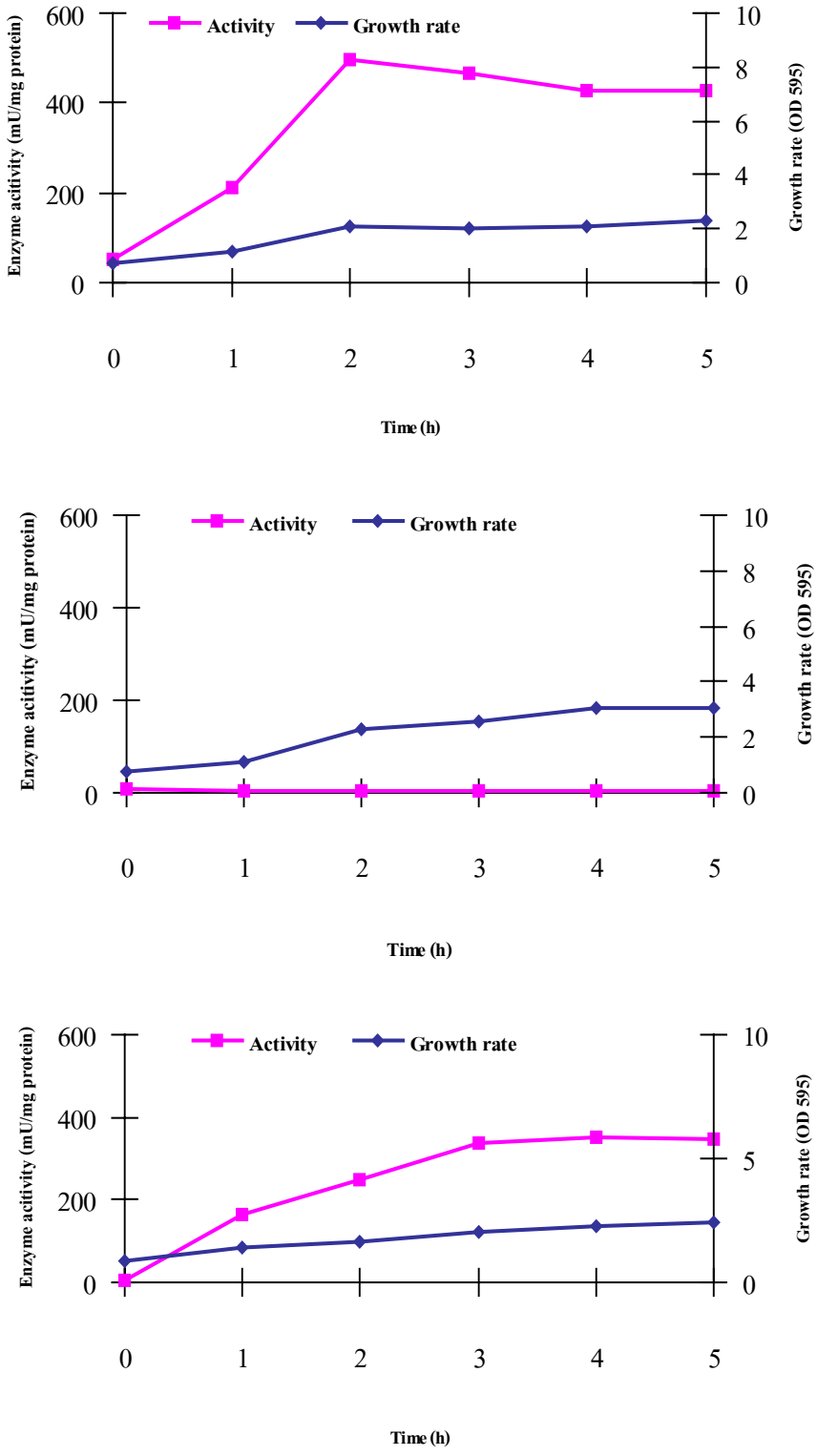


Figure 14. Time course analysis of cholestrol oxidase expression in *E. coli* M15 (pREP4) carrying plasmid (A) pCO117, (B) pQE 30 and (C) pQE 30-choA.

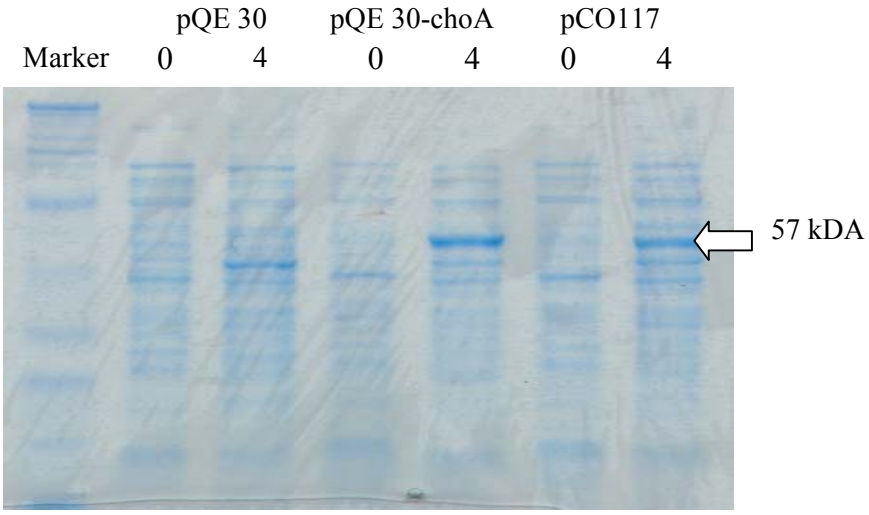


Figure 15. SDS-PAGE of crude cell lysate cholesterol oxidase from M15 (pREP4) carrying various plasmids before induction (0 h) and after induction (4 h).

2.5 Purification of cholesterol oxidase protein

Cholesterol oxidase protein produced by *E. coli* M15 that carried both plasmids (pREP4 and pQE 30-choA) was purified under native condition. The specific activity of purified cholesterol oxidase was 5.59 U mg protein^{-1}. The protein showed a single band on SDS-PAGE with slight difference from commercial *Streptomyces* sp. (Sigma, USA), but the same as that obtained from *E. coli* JM109 pCO117. The molecular weight of commercial cholesterol oxidase produced from *Streptomyces* sp was about 55 kDa with BSA added. For confirmation, 2 % BSA was added in purified cholesterol oxidase protein before electrophoresis on acrylamide gel. The result was shown in Figure 16. In this picture, the top band of lanes 1, 2 and 3 showed BSA that was added in the commercial *Streptomyces* sp cholesterol oxidase. Lane 4 was the crude cell lysate before induction and lane 5 was the crude cell lysate before purification. Lane 6 represented purified cholesterol oxidase protein without BSA, whereas lane 7 with

BSA added. The top bands of lane 7 represented BSA that was added in the protein while the bottom bands were cholesterol oxidase protein. Moreover, the Western blot analysis of purified cholesterol oxidase protein confirmed that this protein could be detected by goat anti-cholesterol oxidase HRP conjugate.

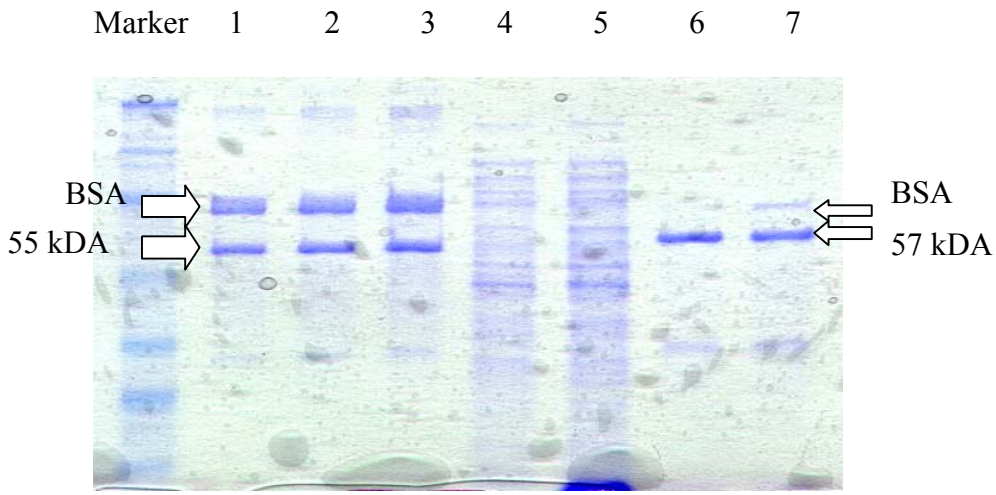


Figure 16. SDS-PAGE of purified cholesterol oxidase protein compared with commercial *Streptomyces* sp. protein. Lane 1, 2, 3 = commercial protein at 3, 5, 7 mg protein; 4, 5 = crude cell lysate before induction and before purification, respectively; 6= 10 μg purified protein; 7= 10 μg purified protein + 2% BSA.

2.6 Factors affecting expression of recombinant plasmids pQE 30-choA

Because of low enzyme activity, unstable clone, and high growth rate were found, it was crucial to study the best condition for expression. Many factors, single or in combination, may have effects on fusion protein. Thus, age of inoculum, medium, proteinase inhibitor, temperature, inoculum density, assay method, and IPTG concentration were studied with the aim to improve enzyme activity.

Because of unstable clone occurred when cultured the recombinant clone in broth medium for 4-5 days. A single colony of M15 (pREP4) pQE 30-choA was inoculated in LB medium supplemented with 25 μg ml^{-1} kanamycin and 100 μg l^{-1} ampicillin and grown at 37°C for 4 h before inoculating in fresh LB medium. The fresh inoculation of this clone showed higher cholesterol oxidase activity (350 mU mg $protein^{-1}$) than an overnight inoculation (221 mU mg $protein^{-1}$) as shown in Table 9. On the contrary, the overnight inoculation of M15 (pREP4) carrying pCO117 gave 7.5-fold cholesterol oxidase activity higher (678 mU mg $protein^{-1}$) than that detected in freshly inoculated clone (90 mU mg $protein^{-1}$). Using fresh inoculum, this bacterium grew slower than using overnight-grown inoculum, but the activity of recombinant plasmid was not as high as demonstrated in the previous case.

Changing the medium to rich nutrition (2xYT medium) gave higher cholesterol oxidase activity (270 mU mg $protein^{-1}$) than that detected from normal LB medium (178 mU mg $protein^{-1}$) as shown in Table 10. Furthermore, the recombinant cells grew rapidly in 2xYT medium.

Both bacterial strains showed slight difference in expression when grown in LB medium plus PMSF as compared to the one without PMSF. With PMSF added, the recombinant plasmids M15 (pREP4) pQE30–choA and pCO117 showed the activity of 405 and 716 mU mg $protein^{-1}$, respectively. Without PMSF both plasmids produced 358 and 638 mU mg $protein^{-1}$, respectively (Table 10).

The recombinant clone was grown at 30°C and 37°C for comparison. At 30°C, higher cholesterol oxidase activity (1,125 mU mg $protein^{-1}$) was observed than that grown at 37°C (834 mU mg $protein^{-1}$) (Table 10).

The assay method also affected enzyme activity. This study found that the method of Allain *et al*. (1974) gave higher activity (589 mU mg protein^{-1}) than that of Masurekar and Goodhue (1978) which showed the activity of 158 mU mg protein^{-1}(Table 10). Moreover, the assay of this enzyme by using Allain's *et al*. (1974) method at 37°C showed higher activity (470 mU mg protein^{-1}) than that assayed at 22°C (279 mU mg protein^{-1}) (Table 10). Thus, the method of Allain *et al*. (1974) may be suitable for *choA* gene from *Streptomyces*, whereas the method of Masurekar and Goodhue (1978) is suitable for assay of cholesterol oxidase from the other sources of bacteria.

Table 10. Factors affecting expression of M15 (pREP4) carrying plasmid pCO117 or pQE 30-choA.

Factors	Enzyme activity (mU/mg protein)	
	pCO117	**pQE 30-choA**
Age of inoculum		
Fresh inoculun	90	350
Overnight-grown inoculum	678	221
Medium		
LB	496	178
2 x YT	–	270
Proteinase inhibitor		
With PMSF	716	405
Without PMSF	638	358
Temperature		
37°C	–	834
30°C	–	1,125
Assay method		
Allain *et al*. (1974)	–	589
Masurekar and Goodhue (1978)	–	158
Allain *et al*. (1974) method		
At 37°C	–	470
At 22°C	–	279

In addition, the density of inoculum also affected the expression of recombinant plasmid. In this study, it was found that the inoculum at 2 ml inoculated in fresh 50 ml medium gave higher activity (1,328 mU mg protein^{-1}) than the recommended concentration, viz 1 ml in fresh 50 ml (845 mU mg protein^{-1}) as shown in Table 11.

Table 11. Density of inoculum on expression of M15 (pREP4) pQE 30-choA.

Inoculum : medium ratio (ml:ml)	Enzyme activity (mU/mg protein)
0.25 : 50	840
1.00 : 50	845
2.00 : 50	1,328
4.00 : 50	1,227

The concentration of IPTG dramatically influenced the expression, thus the optimal concentration of IPTG was established. The result showed that lower or higher IPTG concentration could affect the expression of recombinant plasmid (Table 12). IPTG concentration of 0.1 mM showed high activity (505 mU mg protein^{-1}) after induction for 8 h, but the concentration of 0.5 and 1.0 gave high activity (851 and 853 mU mg protein^{-1}) after induction for 3 and 2 h, respectively. On the other hand, adding IPTG to the final concentration of 1.5, 2.0 and 2.5 mM showed high activity (994, 872 and 815 mU mg protein^{-1}, respectively) after induction for 1 or 2 h before decreasing slowly.

However, the expression of recombinant plasmid pQE 30-choA in *E. coli* M15 (pREP4) was not higher than that of the pCO117 in *E. coli* JM 109. This could be due to the promoter and additional 8 amino acids which resulted from the plasmid construction. Since the constructed clone has ribosome binding site, first ATG, double *lac* operon, 6-histidine sequences that favored *E. coli* and extra 8 amino acids before the *choA* sequences, thus these sequences might be involved in the expression of *Streptomyces choA* gene. The *Streptomyces* gene failed to recognize *E. coli* sequence which resulted in low expression of the enzyme, or the folding structure of cholesterol oxidase protein interfered with those extra amino acids as witnessed by Sambrook *et al*. (2000).

Table 12. The effect of IPTG concentration on expression of M15 (pREP4) pQE 30-choA.

IPTG concentration (mM)	Time after induction (h)	Enzyme activity (mU/mg protein)
0.1	1	290
	2	302
	3	310
	8	505
0.5	1	103
	2	774
	3	851
	8	490

Table 12. Continued.

IPTG concentration (mM)	Time after induction (h)	Enzyme activity (mU/mg protein)
1.0	1	766
	2	853
	3	810
	8	438
1.5	1	994
	2	926
	3	950
	8	484
2.0	1	829
	2	872
	3	843
	8	487
2.5	1	810
	2	815
	3	801
	8	479

CHAPTER V

CONCLUSIONS

1. *Agrobacterium*-mediated transformation in mungbean

This experiment focused on using cotyledonary nodes and cotyledons as explants for *Agrobacterium*-mediated transformation. The average of 31.25% transformation was obtained, but only one GUS-positive shoot could be regenerated. Transformation methods also influenced the production of transformed plant. When using soybean method for transformation, no GUS-positive explants were found as compared to using hairy root method. Moreover, the latter method was less laborious and less bacterial contaminted as compared to the protocol reported previously (Savka *et al*., 1990). This 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. However, only 13.25% of the lines were GUS positive. Not only the wounding technique but also the cultivars, seed age, pre-incubation time, and co-culture conditions were important factors affecting the transformation efficiency. Plant regeneration from the transformed calli was unsuccessful, despite of the recent report on regeneration of mungbean (Tivarekar and Eapen, 2001).

2. Cloning and expression of choleserol oxidase gene (*choA*) in *E. coli* M15 (pREP4)

After Murooka *et al*. (1986) first reported the success in the production of an intracellular cholesterol oxidase from *Streptomyces* in *E. coli*, the cholesterol oxidase gene, *choA*, from plasmid pCO117 was used for studying the expression and purification system in *E. coli* strain M15 (pREP4). This gene, when ligated with the expression vector pQE 30, showed the correct reading frame. The constructed plasmid pQE30-choA, when transformed into *E.coli* M15 (pREP4), revealed an intracellular soluble protein. Time course analysis of this clone showed the highest activity after the cells were grown for 4 h with IPTG and purified protein showed a single band with M_r of 57 kDa. However, the expression of *Streptomyces* cholesterol oxidase gene in this system was not as high as expected. This could be due to the promoter and one additional 8-amino acid sequence that resulted from the plasmid construction. In addition, production of *Streptomyces* cholesterol oxidase enzyme of this recombinant clone appeared to be influenced by inoculum age, medium, proteinase inhibitor, temperature, density of cell, IPTG concentration and assay method. Thus, this constructed plasmid might not be suitable for the expression of *Streptomyces* cholesterol oxidase gene in *E. coli* strain M15 (pERP4).

REFERRENCES

Alam, M. F., Datta, K., Abrigo, E., Oliva, N., Tu, J., Virmani, S. S. and Datta S. K. 1999. Transgenic insect-resistant maintainer line (IR68899B) for improvement of hybrid rice. **Plant Cell Rep**. 18 : 572-575.

Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W. and Fu, P. C. 1974. Enzymatic determination of total serum cholesterol. **Clin. Chem**. 20 : 470-475.

Bean, S. J., Gooding, P. S. and Mullineaux, P. M. 1997. A simple system for pea transformation. **Plant Cell Rep.** 16 : 513-519.

Becker, J., Vogel, T.,Iqbal, J. and Nagl, W. 1994. *Agrobacterium*-mediated transformation of *Phaseolus vulgaris* : adaptation of some conditions. **Annu. Rep. Bean Improve**. Coop. USA 37 : 256-263.

Boulter, D. 1993. Insect pest control by copying nature using genetically engineered crops. **Phytochemistry** 34 : 1453-1466.

Brigidi, P., Bolognani, F., Rossi, M., Cerre, C. and Matteuzzi, D. 1993. Cloning of the gene for cholesterol oxidase in *Bacillus* spp., *Lactobacillus reuteri* and its expression in *Escherichia coli*. **Lett. Appl. Microbiol.** 17 : 61-64.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Ann. Biochem.** 72 :248-254.

Cheng, M., Jarret, R. L., Li, Z., Xing, A. and Demski, J. W. 1996. Production of fertile transgenic peanut (*Arachis hypogaea* L.) plants using *Agrobacterium tumefaciens*. **Plant Cell Rep.** 15 : 653-657.

Cheng, Z. M., Schnurr, J. A., Kapaun, J. A. 1998. Timmentin as an alternative antibiotic for suppression of *Agrobacterium tumefa*ciens in genetic transformation. **Plant Cell Rep.** 17 : 646-649.

Cho, H. J., Choi, K. P., Yamashia, M., Morikawa, H. and Murooka, Y. 1995. Introduction and expression of the *Streptomyces* cholesterol oxidase gene (*ChoA*), a potent insecticidal protein active against boll weevil larvae, into tobacco cells. **Microbiol. Biotechnol.** 44 : 133-138.

Cho, H. J, Widholm, J. M., Tanaka, N., Nakanishi, Y. and Murooka, Y. 1998. *Agrobacterium rhizogenes*-mediated transformation and regeneration of legume *Astragalus sinicus* (Chinese milk vetch). **Plant Sci.** 138 : 53-65.

Cho, H-J., Farrand, S. K., Noel, G. R. and Widholm, J. M. 2000. High-efficiency induction of soybean hairy roots and propagation of the soybean cyst nematode. **Planta** 210 : 195-204.

Christou P. 1990. Morphological description of transgenic soybean chimeras created by the delivery, integration and expression of foreign DNA using electric discharge particle acceleration. **Ann. Bot.** 66 : 379-386.

Christou P. (ed.). 1992. *Vigna* species. ***In* Genetic Engineering and *In Vitro* Culture of Crop Legumes**, pp. 267-278, Pennsylvania : Technomic Publishing.

Corbin, D. R., Greenplate, J. T., Wong, E. Y. and Purcell, J. P. 1994. Cloning of an insecticidal cholesterol oxidase gene and its expression in bacteria and in plant protoplasts. **Appl. Environ. Microbiol.** 60 : 4239-4244.

Corbin, D. R., Greenplate, J. T. and Purcell, J. P. 1998. The identification and development of proteins for control of insects in genetically modified crops. **HortSci.** 33 : 614-617.

Costa, M. G. C., Nogueira, F. T. S., Figueira, M. L., Otoni, W. C., Brommonschenkel, S. H. and Cecon, P. R. 2000. Influence of the antibiotic timentin on plant regeneration of tomato (*Lycopersicon esculentum* Mill.) cultivars. **Plant Cell Rep.** 19 : 327-332.

Damiani, F. and Arcioni, S. 1991. Transformation of *Medicago arborea* L. with an *Agrobacterium rhizogenes* binary vector carrying the hygromycin resistance gene. **Plant Cell Rep.** 10 : 300-303.

De Kathen, A. and Jacobsen, H. J. 1990. *Agrobacterium tumefaciens*-mediated transformation of *Pisum sativum* L. using binary and cointegrate vectors. **Plant Cell Rep.** 9 : 276-279.

Doukya, N. and Aono, R. 1998. Purification of extracellular cholesterol oxidase with high activity in the presence of organic solvents from *Pseudomonas* sp. strain ST-200**. Appl. Environ. Microbiol.** 64 : 1929-1932.

Eapen, S., Kohler, F., Gerdemann, M. and Schieder, O. 1987. Cultivar dependence of transformation rates in moth bean after co-cultivation of protoplasts with *Agrobacterium tumefaciens*. **Theor. Appl. Genet.** 75 : 207-210.

Fontana, G. S., Santini, L., Caretto, S., Frugis, G. and Mariotti, D. 1993. Genetic transformation in grain legume *Cicer arietinum* L. (chickpea). **Plant Cell Rep.** 12 : 194-198.

Franklin, C. I., Trieu, T. N., Cassidy, B. G., Dixon, R. A. and Nelson, R. S. 1993. Genetic transformation of green bean callus via *Agrobacterium*-mediated DNA transfer. **Plant Cell Rep.** 12 : 74-79.

Frary, A. 1995. The use of *Agrobacterium tumefaciens*-mediated transformation in the map-based cloning of tomato genes and an analysis of factor affecting transformation efficiency. **Ph.D. thesis**, **Cornell University**, Ithaca, NY.

Frary, A and Earle, E. D. 1996. An examination of factors affecting the efficiency of *Agrobacterium*-mediated transformation of tomato. **Plant Cell Rep.** 16 : 235-240.

Fujishiro, K., Ota, T., Hasegawa, M., Yamaguchi, K., Mizukami, T. and Uwajima, T. 1990. Isolation and identification of the gene of cholesterol oxidase from *Brevibacterium sterolicum* ATCC 21387, a widely used enzyme in clinical analysis. **Biochem. Biophys. Res. Commun.** 172 : 721-727.

Fukuya, M. and Miyake, Y. 1979. Purification and some properties of cholesterol oxidase from *Schizophyllum commune* with covalently bound flavin. **J. Biochem.** 85 : 1183-1193.

Fullner, K. J. and Nester, E. W. 1996. Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens*. **J. Bacteriol**. 178 : 1498-1504.

Gamborg, O., Miller, R. A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. **Exp. Cell Res.** 50 : 151-158.

Garcia, J. A., Hille, J. and Goldbach, R. 1986. Transformation of cowpea *Vigna unguiculata* cells with an antibiotic resistance gene using a Ti-plasmid derived vector. **Plant Sci.** 44 :37-46.

Garcia, J. A., Hille, J., Vos, P. and Goldbach, R. 1987. Transformation of cowpea *Vigna unguiculata* cells with a full length DNA copy of cowpea mosaic virus mRNA. **Plant Sci.** 48 : 89-98.

Gatehouse, A. M. R. and Hilder, V. A. 1994. Genetic manipulation of crops for insect resistance. ***In* Molecular Biology in Crop Protection** (eds. G. Marshall and W. Dale), pp. 177-201, London : Chapman & Hall.

Gatehouse, A. M. R., Shi, Y., Powell, K. S., Brough, C., Hilder, V. A., Hamilton, W. D. O., Newell, C. A., Merryweather, A., Boulter, D. and Gatehouse, J. A. 1993. Approaches to insect resistance using transgenic plants. **Phil. Trans. Res. Soc. Land. B.** 279-286.

Godwin, I., Todd, G., Ford-Lloyd, B. and Newbury, H. J. 1991. The effects of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. **Plant Cell Rep.** 9 : 671-675.

Golds, T. J., Lee, J. Y., Husnain, T., Ghose, T. K. and Davey, M. R. 1991. *Agrobacterium rhizogenes*-mediated transformation of the forage legumes *Medicago sativa* and *Onobrychis vicifolia*. **J. Exp. Bot.** 42 : 1147-1157.

Gordon-Kamm, W. J., Spencer, M. T., Mangano, M. L., Adams, T. R., Daines, R. J., Start, W. G., O'Brien, J. V., Chambers, S. A., Adams Jr., W. R., Willets, N. R., Rice, T. B., Mackey, C. J., Krueger, R. W., Kausch, A. P. and Lemaux, P. G. 1990. Transformation of maize cells and regeneration of fertile transgenic plants. **Plant Cell** 2 : 603-618.

Goswami, J., Sanyal, R., Mohan, J., Dhawan, V. and Lakshmikumaran, M. 1998. Genetic manipulation of crops for insect resistance. ***In*** **Plant Tissue Culture and Molecular Biology : Applications and Prospects** (ed. P. S. Srivastava), pp 670-693, India : Narsosa Publishing House.

Greenplate, J. P., Duck, N. B., Pershing, J. C. and Purcell, J. P. 1995. Cholesterol oxidase : an oostatic and larvicidal agent active against the cotton boll weevil, *Anthonomus grandis*. **Entomol. Exp. Appl.** 74 : 253-258.

Hidaka, T., Omura, M., Ukagi, M., Tomiyama, M., Kato, A., Ohshima, M. and Motoyoshi, F. 1990. *Agrobacterium*-mediated transformation and regeneration of *Citrus* spp. from suspension cells. **Jpn. J. Breed.** 40 : 199-207.

Hinchee, M., Connor-Ward, D. V., Newell, C. A., McDonnell R. E., Sato, S. J., Gasser, C. S., Fischhoff, D. A., Re, D. B., Fraley, R. T. and Horsch, R. B. 1998. Production of transgenic soybean plants using *Agrobactrium*-mediated DNA transfer. **Bio/Technol.** 6 : 915-922.

Hood, E. E., Jen, G., Kayes, L., Kramar, L., Fraley, R. T. and Chilton, M. D. 1984. Restriction endonuclease map of pTiBo542, a potential Ti-plasmid vector for genetic engineering of plants. **Bio/Technol.** 2 : 702-709.

Hood, E. E., Gelvin, S. B., Melchers, L. S. and Hoekema, A. 1993. New *Agrobacterium* helper strains for gene transfer to plants. **Transgenic Res**. 2 : 208-218.

Horii, M., Ishizaki, T., Paik, S. Y., Manome, T. and Murooka, Y. 1990. An operon containing the genes for cholesterol oxidase and a cytochrome P-450-like protein from a *Streptomyces* sp. **J. Bacteriol.** 172 : 3644-3653.

Inouye, Y., Taguchi, K., Fujii, A., Ishimaru, K., Nakamura, S. and Nomi, R. 1982. Purification and characterization of extracellular 3β-hydroxysteroid oxidase produced by *Streptoverticillium cholesterolicum*. **Chem. Pharm. Bull.** 30 : 951-958.

Ishizaki, T. Hirayama, N., Shinkawa, H., Nimi, o. and Murooka, Y. 1989. Nucleotide sequence of the gene for cholesterol oxidase from a *Streptomyces* sp**. J. Bacteriol.** 171 : 596-601.

Jaiwal, P. K., Kumari, R., Ignacimuthu, S., Potrykus, I and Sautter, C. 2001. *Agrobacterium tumefaciens*-mediated genetic transformation of mungbean (*Vigna radiata* L. Wilczek)-a recalcitrant grain legume. **Plant Sci.** 16 : 239-247.

Jin, S., Komari, T., Gordon, M. P. and Nester, E. W. 1987. Genes responsible for the supervirulence phenotype of *Agrobacterium tumefaciens* AA281. **J. Bacteriol.** 169 : 4417-4425.

Joersbo, M. and Biunsledt, J. 1992. Sonication : a new method for gene transfer to plants. **Plant Physiol.** 85 : 230-234.

Johnson, T. L. and Somkuti, G. A. 1991. Isolation of cholesterol oxidase from *Rhodococcus equi* ATCC 33706. **Biotechnol. Appl. Biochem.** 13 : 196-204.

Jovanin, L., Bottino, M. B., Girard, C., Morrot, G. and Giband, M. 1998. Transgenic plants for insect resistance : Review. **Plant Sci.** 131 : 1-11.

Judith-Webb, K., Jones, S., Robbins, M. and Minchin, F. 1990. Characterzation of transgenic root cultures of *Trifolium repens*, *Trifolium pratense* and *Lotus corniculatus* and transgenic plants of *Lotus corniculatus*. **Plant Sci.** 70 : 243-254.

Karthikeyan, A. S., Sarma, K. S. and Veluthambi, K. 1996. *Agrobacterium tumefaciens*-mediated transformation of *Vigna mungo* (L.) Hepper. **Plant Cell Rep.** 15 : 328-331.

Kenney, W. C., Singer, T. P., Fukuyama, M. and Miyake, Y. 1979. Identification of the covalently bound flavin prosthetic group of cholesterol oxidase. **J. Biol. Chem.** 254 : 4689-4690.

Kononowicz, A. K., Narasimhan, M. L., Reuveni, M. McClatchey, G., Bressan, P. H., Zhang, Y., Larosa, P. C., Murdock, L. L., Chrispeels, M. J., Bressan, R. A. and Hasegawa, P. M. 1993. Genetic transformation of cowpea (*Vigna unguiculata*) using microprojectile bombardment and *Agrobacterium tumefaciens* infection. **Plant Physiol.** 102:Suppl., abstract no. 945.

Kumar, V., Jones, B. and Davey, M. R. 1991. Transformation by *Agrobacterium rhizogenes* and regeneration of transgenic shoots of the wild soybean *Glycine argyrea*. **Plant Cell Rep.** 10 : 135-138.

Kudirka, D. T., Colburn, S. M., Hinchee, M. A. and Wright, M. S. 1986. Interactions of *Agrobacterium tumefaciens* with soybean (*Glycine max* L. Merr.) leaf explants in tissue cultures. **Can. J. Genet. Cytol.** 28 : 808-817.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature** 227 : 680-685.

Lee, S. Y., Rhee, H. I., Tae, W. C., Shin, J. C. and Park, B. K. 1989. Purification and characterization of cholesterol oxidase from *Pseudomonas* sp. and taxonomic study of the strain. **Appl. Microbiol. Biotechnol.** 31 : 542-546.

Lewis, M. E. and Bliss, F. A. 1994. Tumor formation and beta-glucuronidase expression in *Phaseolus vulgaris* inoculation with *Agrobacterium tumefaciens***. J. Amer. Soc. Hort. Sci**. 119 : 361-366.

Ling, H. Q., Kriseleit, D. and Ganal, M. W. 1998. Effect of ticarcillin potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* Mill.) **Plant Cell Rep.** 17 : 843-847.

Manners, J. M. and Way, H. 1989. Efficient transformation with regeneration of tropical pasture legume *Stylosanthes humilis* using *Agrobacterium rhizogenes* and a Ti plasmid-binary vector system. **Plant Cell Rep.** 8 : 341-345.

Mansur, E. A., Lacorte, C., De Freitas, V. G., De Oliveira, D. E., Timmerman, B. and Cordeiro A. R. 1993. Regulation of transformation efficiency of peanut (*Arachis hypegaea* L.) explants by *Agrobacterium tumefaciens*. **Plant Sci.** 89 : 93-99.

Mariotti, D., Fontana, G. S. and Santini, L. 1989. Genetic transformation of grain legumes; *Phaseolus vulgaris* L. and *P. coccineus* L. **J. Genet. Breed.** 43 : 77-82.

Masurekar, P. S. and Goodhue, C. T. 1978. **United States patent No.4,093,517**, Eastman Kodak Company,Rochester, NY.

Meurer, C. A., Dinkins, R. D. and Collins, G. B. 1998. Factors affecting soybean cotyledon node transformation. **Plant Cell Rep.** 18 : 180-186.

Murooka, Y., Ishizaki, T., Nimi, O. and Maekawa, N. 1986. Cloning and expression of a *Streptomyces* cholesterol oxidase gene in *Streptomyces lividans* with plasmid pIJ702. **Appl. Environ. Microbiol.** 52 : 1382-1385.

Muthukumar, B., Mariamma, M., Veluthambi, K. and Gnanam, A. 1996. Genetic transformation of cotyledon explants of cowpea (*Vigna unguiculata* L. Walp) using *Agrobacterium tumefaciens*. **Plant Cell Rep.** 15 : 980-985.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiol. Plant.** 15 : 473-497.

Nagl, W., Isgnacimuthu, S. and Becker, J. 1997. Genetic engineering and regeneration of *Phaseolus* and *Vigna*: state of the art and new attempts. **J. Plant Physiol.** 150 : 625-644.

Nagl, W. and Ehemann, A. 1994. Transformation of protoplasts from asparagus bean, *Vigna unguiculata* ssp. *sesquiedalis* L., and attempts of regeneration. **Plant Physiol.** 13 : 177-182.

Nauerby, B., Billing, K. and Wyndaele, R. 1997. Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentration suitable for elimination of *Agrobacterium tumefaciens*. **Plant Sci.** 123 : 169-177.

Nomura, N., Choi, K. P., Yamashita, M., Yamamoto, H. and Murooka, Y. 1995. Genetic modification of the *Streptomyces* cholesterol oxidase gene for expression in *Escherichia coli* and development of promoter-probe vectors for use in enteric bacteria. **J. Ferment. Bioeng.** 79 : 410-416.

Ohta, T., Fujishiro, K., Yamaguchi, K. Tamura, Y., Aisaka, K., Uwajima, T. and Hasegawa, M. 1991. Sequence of gene *choB* ending cholesterol oxidase of *Brevibacterium sterolicum*: comparison with *choA* of *Streptomyces* sp. SA-COO. **Gene** 103 : 93-96.

Ohta, T., Fujishiro, K., Yamaguchi, K., Uwajima, T., Aisaka, K. and Hasegawa, M. 1992. Hyperexpression and analysis of choB encoding cholesterol oxidase of

Brevibacterium sterolicum in *Escherichia coli* and *Streptomyces lividans*. **Biosci. Biotech. Biochem.** 56 : 1786-1791.

Owens, L. D. and Cress, D. E. 1985. Genotypic variability of soybean response to *Agrobacterium* strains harboring the Ti or Ri plasmids. **Plant Physiol.** 77 : 87-94.

Peferon, M. 1997. Progress and prospects for field use of *Bt* genes in crops. **TIBTECH** 15 : 173-177.

Penza, R., Lurquin, P. F. and Fillipone, E. 1991. Gene transfer by co-cutlivation of mature embryos with *Agrobacterium tumefaciens* :Application to cowpea (*Vigna unguiculata* Walp.). **Plant Physiol.** 138 : 39-42.

Perlak, F. J., Deaton, R. N., Armstrong, T. A., Sims, S. R., Greenplate, J. T., Fishchoff, D. A. 1990. Insect resistant cotton. **Biotechnology** 8 : 939-943.

Petit, A., Stougaard, J., Kuhle, A., Marcker, K. A. and Tempe, J. 1987. Transformation and regeneration of the legume *Lotus corniculatus*: a system for molecular studies of symbiotic nitrogen fixation. **Mol. Gen. Genet.** 207 : 245-250.

Pigeaire, A., Abernethy, D., Smith, P. M., Simpson, K., Fletcher, N., Lu, C. Y., Atkins C. A. and Cornish, E. 1997. Transformation of a grain legume (*Lupinus angustifolius* L.) via *Agrobacterium tumefaciens*-mediated gene transfer to shoot apices. **Mol. Breed.** 3 : 341-349.

Puonti-Kaerlas, J., Eriksson, T. and Engstrom, P. 1990. Production of transgenic pea (*Pisum sativum* L.) plants by *Agrobacterium tumefaciens*-mediated gene transfer. **Theor. Appl. Genet.** 80 : 246-252.

Purcell, J. P., Greenplate, J. T., Jennings, M. G., Ryerse, J. J., Pershings, J. C., Sims, S. R., Prinsen, M. J., Corbin, D. R., Tran, M., Sammons, R. D. and Stonard, R. J. 1993. Cholesterol oxidase: A potent insecticidal protein active against boll weevill larvae. **Biochem. Biophys. Res. Commun.** 196 : 1406-1413.

Rech, F. L., Golds,T. J., Husnain, T., Vainstein, M. H., Jones, B., Hammatt, N., Mulligan, B. J. and Davey, M. R. 1989. Expression of a chimaric kanamycin resistance gene introduced into the wild soybean *Glycine canescens* using a cointegrate Ri plasmid vector. **Plant Cell Rep.** 8 : 33-36.

Saitou, T., Kamada, H. and Harada, H. 1992. Involvement of phytohormones in light-induced adventitious shoot formation of horseradish hairy roots. **Plant Sci.** 86: 161-166.

Sambrook, J., Fritsch, E. F. and Manniatis T. 2000. **Molecular cloning: a laboratory manual.** Cold Spring Harbor Laboratory Press, New York.

Sampson, N. S. and Chen, X. 1998. Increased expression of *Brevibacterium sterolicum* cholesterol oxidase in *Escherichia coli* by genetic modification. **Protein Exp. Purif.** 12 : 347-352.

Santarem, E. R., Trick, H. N., Essig, J. S. and Finer, J. J. 1998. Sonication-assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: optimization of transient expression. **Plant Cell Rep.** 17 : 752-759.

Sarikarin, N., Srinives, P., Kaveta, R. and Saksoong, P. 1999. Effect of seed texture layer on bruchid infestation in mungbean *Vigna radiata* (L.) Wilczek. **ScienceAsia** 25 : 203-206.

Savka, M. A., Ravillion, B., Noel, G. R. and Farrand, S. K. 1990. Induction of hairy roots on cultivated soybean genotypes and their use to propagate the soybean cyst nematode. **Phytopathology** 80 : 503-508.

Schroeder, H. E., Schotz, A. H., Wardley-Richardson, T., Spencer, D. and Higgins, T. J. V. 1993. Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.). **Plant Physiol.** 101 : 751-757.

Schuler, T. H., Poppy, G. M., Kerry, B. R. and Denholm, I. 1998. Insect-resistant transgenic plants. **TIBTECH** 16 : 168-175.

Solaiman, D. K. Y. and Somkuti, G. A. 1991. Expression of streptomycete cholesterol oxidase in *Escherichia coli*. **J. Ind. Microbiol.** 8 : 253-258.

Somkuti, G. A. Solaiman, D. K. Y., Johson, T. L. and Steinberg, D. H. 1991. Transfer and expression of a *Streptomyces* cholesterol oxidase gene in *Streptococcus thermophilus*. **Biotechnol. Appl. Biochem.** 13 : 238-245.

Somkuti, G. A., Solaiman, D. K. Y. and Steinberg, D. H. 1992. Expression *of Streptomyces* sp. cholesterol oxidase in *Lactobacillus casei*. **Appl. Microbiol. Biotechnol.** 37 : 330-334.

Spano, L., Mariotti, D., Pezzotti, M., Damiani, F. and Arcioni, S. 1987. Hairy root transformation in alfalfa (*Medicago sativa* L.). **Theor. Appl. Genet.** 73: 523-530.

Talekar, N. S. and Chen, B. S. 1983. Seasonality of insect pests of soybean and mungbean in Taiwan. **Entomological Society of America** 76 : 34-37.

Tivarekar, S. and Eapen, S. 2001. High frequency plant regeneration from immature cotyledons of mungbean. **Plant Cell, Tiss. Org. Cult.** 66 : 227-230.

The QIAexpressionist TM. 2000. **A Handbook for High-Level Expression and Purification of 6xHis-Tagged Protein**. (4th eds). QIAGEN, Valencia CA, USA.

Thomas, J. C., Adams, D. G., Nessler, C. L., Brown, J. K. and Bohnert, H. J. 1995. Tryptophan decarboxylase, tryptamone, and reproduction of the whitefly. **Plant Physiol.** 109 : 717-720.

Uwajima, T., Yagi, H., Nakamura, S. and Terada, O. 1973. Isolation and crystallization of extracellular 3β-hydroxysteroid oxidase of *Brevibacterium sterolicum* nov. sp**. Agric. Biol. Chem.** 37 : 2345-2350.

Widholm, J. M. 1995. Leguminous plants. ***In*** **Transformation of Plants and Soil Microorganisms** (eds. K. Wang, A. Herrera-Estrella and M. Van montagu). pp. 101-124, Cambridge : Cambridge University Press.

Yamada, T., Teraishi, M., Hattori, K. and Ishimoto, M. 2001. Transformation of azuki bean by *Agrobacterium tumefaciens*. **Plant Cell, Tiss. Org. Cult.** 64 : 47-54.

Yazdi, M. T., Zahraei, M., Aghaepour, K. and Kumaranpour, N. 2001. Purification and partial characterization of cholesterol oxidase from *Streptomyces fradiae*. **Enzyme Microb. Technol.** 18 : 410-141.

Yan, B., Readdy, M. S. S., Collins, G. B. and Dinkins, R. D. 2000. *Agrobacterium tumefaciens*-mediated transformation of soybean [*Glycine max* (L.) Merr.] using immature zygotic cotyledon explants. **Plant Cell Rep.** 19 : 1090-1097.

Zimmerman, T. W. 1995. Effect of timentin for controlling *Agrobacterium tumefaciens* following cocultivation on select plant species. ***In Vitro*** **Cell. Dev. Biol.** 31 : 70A.

Zhang, Z., Coyne, D. P. and Mitra, A. 1997. Factors affecting *Agrobacterium*-mediated transformation of common bean. **J. Amer. Soc. Hort. Sci.** 122 : 300-305.

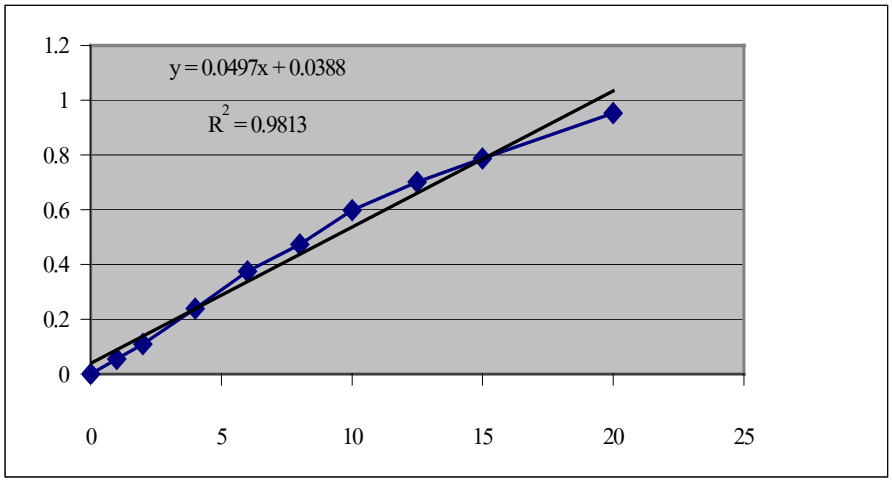
APPENDIX

APPENDIX A

Determination of target protein

1. BSA standard curve

BSA concentration (μg/(μl)	A_{595} nm
0	0
1	0.055
2	0.110
4	0.238
6	0.374
8	0.473
10	0.598
12.5	0.702
15	0.788
20	0.953



2. Calculation of protein concentration

Sample	A_{595} nm	X Value (µg/10 ul)	X/10 (µg/µl)	protein amount (µg/ml)
Intercellular without IPTG				
M15 pCO117	0.484	8.959	0.896	895.9
M15 pQE 30	0.338	6.028	0.602	602.8
M15 pQE 30-choA	0.451	8.307	0.830	830.7
M15 pQE 31	0.437	8.014	0.801	801.4
M15 pQE 31-choA	0.493	9.140	0.914	914.0
M15 pQE 32	0.508	9.442	0.944	944.2
M15 pQE 32-choA	0.494	9.169	0.917	916.9
Intercellular with IPTG				
M15 pCO117	0.547	10.23	1.023	1023
M15 pQE 30	0.609	11.48	1.148	1148
M15 pQE 30-choA	0.444	8.169	0.816	816.9
M15 pQE 31	0.562	10.54	1.054	1054
M15 pQE 31-choA	0.547	10.23	1.023	1023
M15 pQE 32	0.633	11.96	1.196	1196
M15 pQE 32-choA	0.566	10.60	1.060	1060

2. Calculation of protein concentration (continued).

Sample	A_{595} nm	X Value (μg/10 ul)	X/10 (μg/μl)	protein amount (μg/ml)
Extracellular without IPTG				
M15 pCO117	0.013	-0.509	-0.050	-50.90
M15 pQE 30	0.010	-0.573	-0.057	-57.34
M15 pQE 30-choA	0.030	-0.837	-0.183	-98.70
M15 pQE 31	0.007	-0.637	-0.063	-63.78
M15 pQE 31-choA	0.010	-0.579	-0.057	-57.94
M15 pQE 32	0.008	-0.611	-0.061	-61.16
M15 pQE 32-choA	0.013	-0.517	-0.051	-51.71
Extracellular with IPTG				
M15 pCO117	0.004	-0.698	-0.069	-69.81
M15 pQE 30	0.015	-0.484	-0.048	-48.49
M15 pQE 30-choA	0.006	-0.647	-0.064	-64.78
M15 pQE 31	0.000	-0.770	-0.077	-77.06
M15 pQE 31-choA	0.016	-0.444	-0.044	-44.46
M15 pQE 32	0.015	-0.474	-0.047	-47.48
M15 pQE 32-choA	0.012	-0.539	-0.053	-53.92

3. Calculation of enzyme activity

Sample	A_{500} nm	A_{500} nm control	A observed- A control	Enzyme activity (U/ml)	Protein (μg/ml)	Specific activity (mU/mg protein)
Intercellular without IPTG						
M15 pCO117	0.215	0.006	0.209	123.3	895.9	137.6
M15pQE30	0.014	0.006	0.008	4.897	602.8	8.123
M15pQE30-choA	0.452	0.006	0.446	263.4	830.7	317.1
M15pQE31	0.015	0.006	0.009	5.664	801.4	7.067
M15pQE31-choA	0.017	0.006	0.010	6.431	914.0	7.035
M15pQE32	0.015	0.006	0.009	5.310	944.2	5.623
M15pQE32-choA	0.020	0.006	0.014	8.555	916.9	9.330
Intercellular with IPTG						
M15 pCO117	0.978	0.006	0.972	573.5	1023	560.2
M15pQE30	0.023	0.006	0.017	10.44	1148	9.089
M15pQE30-choA	0.370	0.006	0.363	214.7	816.9	262.8
M15pQE31	0.023	0.006	0.017	10.38	1054	9.859
M15pQE31-choA	0.119	0.006	0.113	67.08	1023	65.56
M15pQE32	0.023	0.006	0.017	10.26	1196	8.581
M15pQE32-choA	0.136	0.006	0.130	77.05	1060	72.64
Extracellular without IPTG						
M15 pCO117	-0.000	0.006	-0.006	-3.776	-50.91	*
M15pQE30	-0.002	0.006	-0.008	-4.779	-57.34	
M15pQE30-choA	0.000	0.006	-0.005	-3.481	-98.70	
M15pQE31	-0.002	0.006	-0.008	-4.838	-63.78	
M15pQE31-choA	-0.001	0.006	-0.007	-4.484	-57.95	
M15pQE32	-0.001	0.006	-0.007	-4.543	-61.17	
M15pQE32-choA	-0.001	0.006	-0.007	-4.307	-51.71	

*Since, no enzyme activity could be detected from this experiment, enzyme specific activity was not calculated.

3. Calculation of enzyme activity (continued)

Sample	A_{500} nm	A_{500} nm control	A observed- A control	Enzyme activity (U/ml)	Protein (μg/ml)	Specific activity (mU/mg protein)
Extracellular with IPTG						
M15 pCO117	-0.010	0.006	-0.016	-9.558	-69.82	*
M15pQE30	-0.009	0.006	-0.015	-9.263	-48.49	
M15pQE30-choA	-0.008	0.006	-0.015	-8.850	-64.79	
M15pQE31	-0.010	0.006	-0.016	-9.912	-77.06	
M15pQE31-choA	-0.010	0.006	-0.016	-9.853	-44.47	
M15pQE32	-0.010	0.006	-0.016	-9.558	-47.48	
M15pQE32-choA	-0.010	0.006	-0.016	-9.617	-53.92	

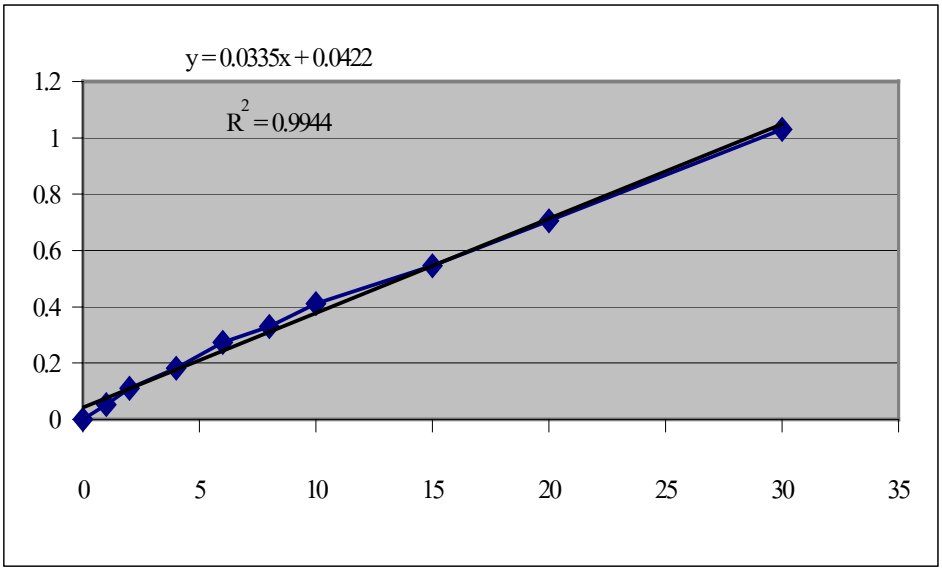
*Since, no enzyme activity could be detected from this experiment, enzyme specific activity was not calculated.

APPENDIX B

Time course analysis of expression

1. BSA standard curve

BSA concentration (μg/(μl)	A_{595} nm
0	0.000
1	0.052
2	0.110
4	0.182
6	0.274
8	0.330
10	0.411
15	0.545
20	0.705
30	1.030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1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35

2. Calculation of protein concentration

Time after induction (h)	Growth rate	A_{595} nm	X Value (μg/10 ul)	X/10 (μg/μl)	protein amount (μg/ml)
M15 (pREP4) pCO117					
0	0.706±0.23	0.161	3.567	0.356	356.7
1	1.174±0.21	0.500	13.66	1.366	1366
2	2.107±0.27	0.452	12.23	1.223	1223
3	1.998±0.14	0.594	16.47	1.647	16472
4	2.100±0.16	0.693	19.44	1.944	1944
5	2.223±0.29	0.709	19.91	1.991	1991
M15 (pREP4) pQE 30					
0	0.732±0.28	0.156	3.408	0.340	340.8
1	1.141±0.19	0.486	13.26	1.326	1326
2	2.276±0.25	0.518	14.22	1.422	1422
3	2.390±0.21	0.860	24.42	2.442	2442
4	2.569±0.14	0.828	23.47	2.347	2347
5	3.072±0.22	0.9398	26.7940	2.6794	2679
M15 (pREP4) pQE 30-choA					
0	0.884±0.20	0.491	13.41	1.341	1341
1	1.407±0.24	0.515	14.13	1.413	1413
2	1.161±0.18	0.574	15.88	1.588	1588
3	2.002±0.26	0.585	16.22	1.623	1622
4	2.263±0.17	0.730	20.53	2.053	2053
5	2.454±0.22	0.795	22.49	2.249	2249

3. Calculation of enzyme activity

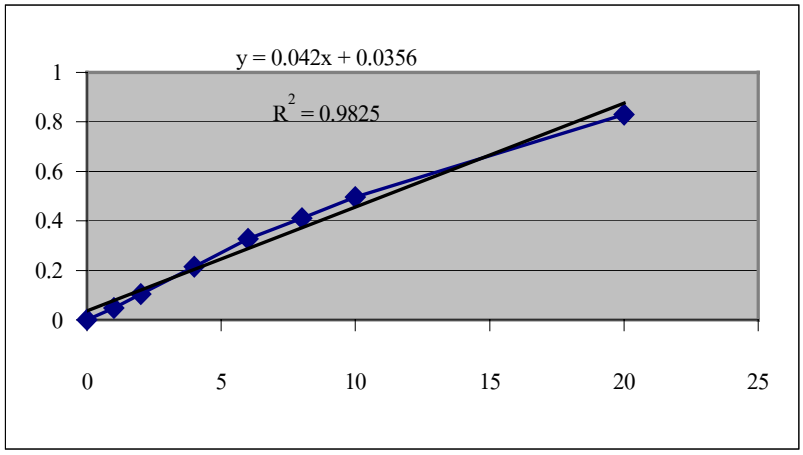
Time after induction (h)	A_{500} nm	A_{500} nm control	A observed- A control	Enzyme activity (U/ml)	Protein (μg/ml)	Specific activity (mU/mg protein)
M15 (pREP4) pCO117						
0	0.029	-0.002	0.031	0.018	356.7	52.76
1	0.487	-0.002	0.490	0.289	1366	211.8
2	1.025	-0.002	1.028	0.606	1223	495.5
3	1.299	-0.002	1.302	0.768	1647	466.1
4	1.413	-0.002	1.416	0.835	1944	429.5
5	1.438	-0.002	1.441	0.8	1991	426.8
M15 (pREP4) pQE 30						
0	0.002	-0.002	0.005	0.003	340.8	9.692
1	0.011	-0.002	0.014	0.008	1326	6.403
2	0.013	-0.002	0.016	0.009	1422	6.882
3	0.023	-0.002	0.026	0.015	2442	6.279
4	0.019	-0.002	0.022	0.013	2347	5.579
5	0.027	-0.002	0.030	0.017	2679	6.605
M15 (pREP4) pQE 30-choA						
0	0.010	-0.002	0.013	0.008	1341	5.980
1	0.387	-0.002	0.390	0.230	1413	163.1
2	0.661	-0.002	0.664	0.391	1588	246.6
3	0.925	-0.002	0.928	0.547	1622	337.6
4	1.218	-0.002	1.221	0.720	2053	350.8
5	1.314	-0.002	1.316	0.776	2249	345.3

APPENDIX C

Purification of cholesterol oxidase protein in large scale

1. BSA standard curve

BSA concentration (µg/(µl)	A_{595} nm
0	0
1	0.048
2	0.103
4	0.213
6	0.326
8	0.411
10	0.496
20	0.830



2. Calculation of protein concentration

Samples	A_{595} nm	X Value (μg/10 ul)	X/10 (μg/μl)	protein amount (μg/ml)
I	0.078	1.009	0.100	100.9
II	0.079	0.753	0.075	75.38
III	0.070	0.650	0.065	65.02

3. Calculation of enzyme activity

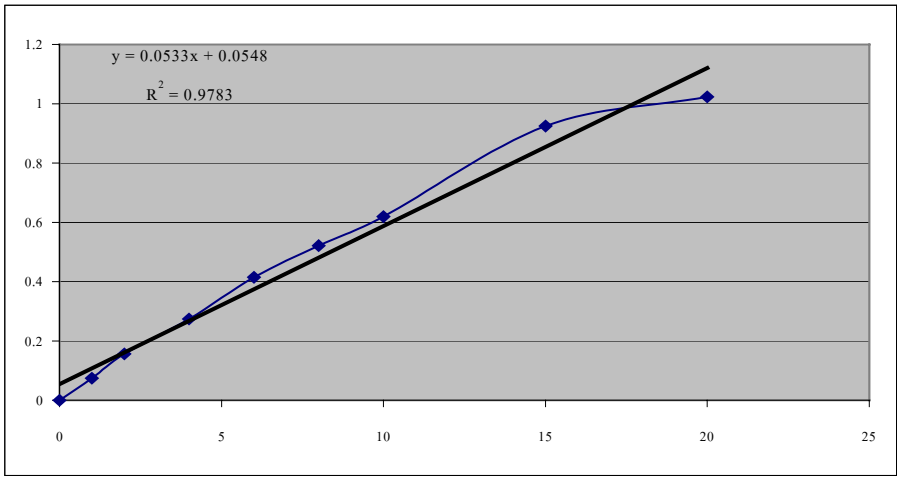
Samples	A_{500} nm	A_{500} nm control	A observed- A control	Enzyme activity (U/ml)	Protein (μg/ml)	Specific activity (U/mg protein)
I	0.957	0.000	0.957	0.564	100.9	5.590
II	0.326	0.041	0.284	0.168	75.38	2.228
III	0.401	0.001	0.399	0.235	65.00	3.627

APPENDIX D

Age of inoculum

1. BSA standard curve

BSA concentration (μg/(μl)	A_{595} nm
0	0
1	0.074
2	0.156
4	0.274
6	0.414
8	0.521
10	0.619
15	0.924
20	1.023



2. Calculation of protein concentration

Age of inoculum	A_{595} nm	X Value (µg/10 ul)	X/10 (µg/µl)	protein amount (µg/ml)
Fresh inoculum				
pCO 117	0.156	3.408	0.340	340.8
pQE 30-choA	0.730	20.534	2.053	2053
Overnight inoculum				
pCO 117	0.653	11.23	1.123	1123
pQE 30-choA	0.246	3.591	0.359	359.0

3. Calculation of enzyme activity

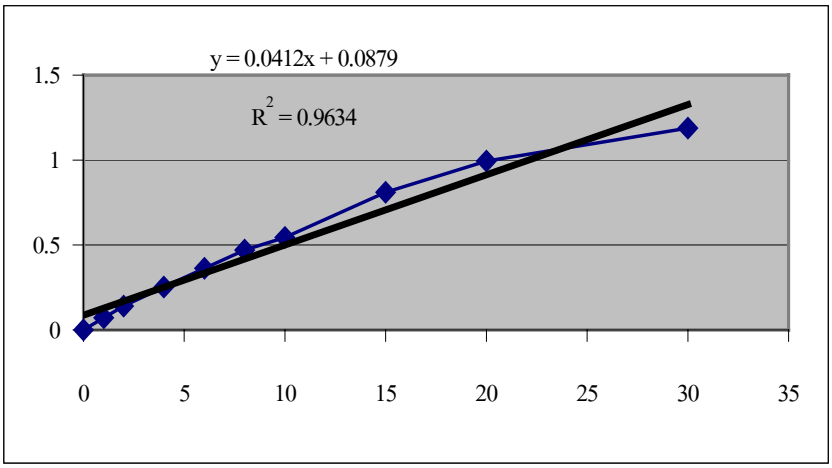
Age of inoculum	A_{500} nm	A_{500} nm control	A observe- A control	Enzyme activity (U/ml)	Protein (µg/ml)	Specific activity (mU/mg protein)
Fresh inoculum						
pCO 117	0.005	-0.000	0.005	0.003	340.8	90.05
pQE 30-choA	1.222	-0.000	1.222	0.720	2053	350.8
Overnight inoculum						
pCO 117	1.291	-0.000	1.291	0.762	1123	678.2
pQE 30-choA	0.134	-0.000	0.134	0.079	359.0	221.6

APPENDIX E

Medium

1. BSA standard curve

BSA concentration (μg/(μl)	A_{595} nm
0	0.000
1	0.072
2	0.141
4	0.253
6	0.363
8	0.469
10	0.545
15	0.809
20	0.995
30	1.188



2. Calculation of protein concentration

hMedium	A_{595} nm	X Value (µg/10 ul)	X/10 (µg/µl)	protein amount (µg/ml)
LB				
pCO 117	0.789	17.02	1.702	1702
pQE 30-choA	0.492	9.81	0.981	981.0
2 x YT				
pQE 30-choA	0.879	19.22	1.922	1922

3. Calculation of enzyme activity

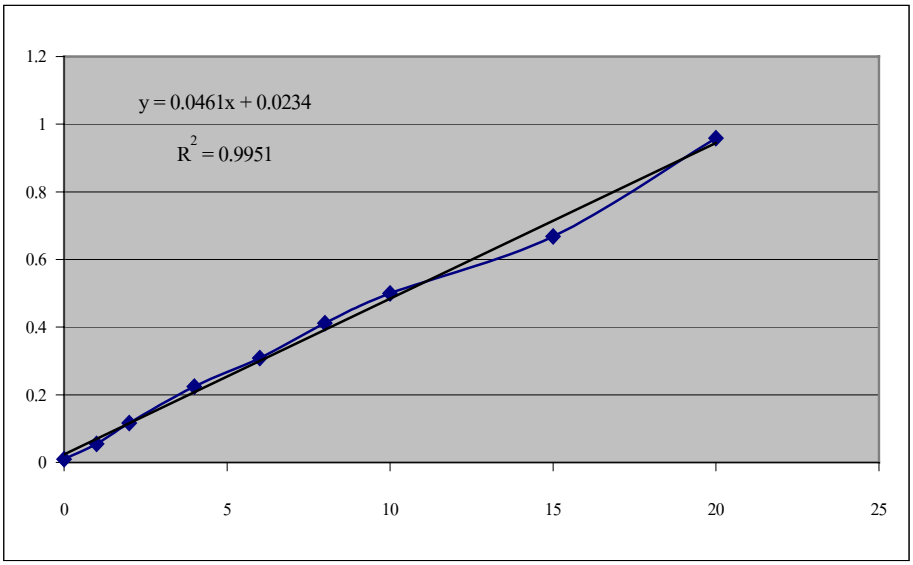
Medium	A_{500} nm	A_{500} nm control	A observed- A control	Enzyme activity (U/ml)	Protein (μg/ml)	Specific activity (mU/mg protein)
LB						
pCO 117	1.433	-0.000	1.433	0.845	1702	496.6
pQE 30-choA	0.296	-0.000	0.297	0.175	981.0	178.6
2 x YT						
pQE 30-choA	0.879	-0.000	0.883	0.519	1922	270.1

APPENDIX F

Proteinase inhibitor

1. BSA standard curve

BSA concentration (μg/(μl)	A_{595} nm
0	0.009
1	0.054
2	0.116
4	0.224
6	0.308
8	0.411
10	0.499
15	0.668
20	0.958



2. Calculation of protein concentration

Sample	A_{595} nm	X Value (µg/10 ul)	X/10 (µg/µl)	protein amount (µg/ml)
Without PMSF				
pCO 117	0.427	8.770	0.877	877.0
pQE 30-choA	0.281	8.960	0.896	896.0
With PMSF				
pCO 117	0.436	5.603	0.560	560.3
pQE 30-choA	0.287	5.718	0.571	571.8

3. Calculation of enzyme activity

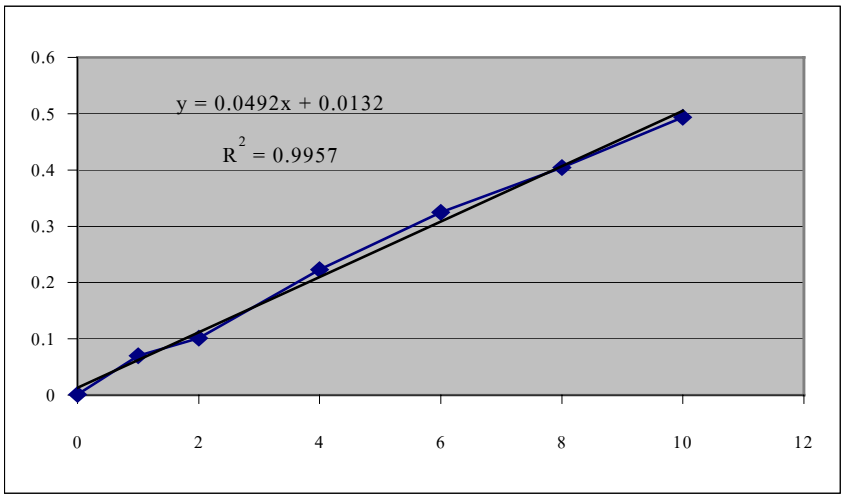
Method	A_{500} nm	A_{500} nm control	A observed- A control	Enzyme activity (U/ml)	Protein (μg/ml)	Specific activity (mU/mg protein)
Without PMSF						
pCO 117	0.949	-0.000	0.949	0.560	877.0	638.8
pQE 30-choA	1.088	-0.000	1.088	0.642	896.0	358.6
With PMSF						
pCO 117	0.340	-0.000	0.340	0.200	560.3	716.7
pQE 30-choA	0.393	-0.000	0.393	0.232	571.8	405.9

APPENDIX G

Temperature

1. BSA standard curve

BSA concentration (μg/(μl)	A_{595} nm
0	0.001
1	0.069
2	0.101
4	0.222
6	0.324
8	0.404
10	0.493
20	0.842
40	1.231



2. Calculation of protein concentration

Sample	A_{595} nm	X Value (µg/10 ul)	X/10 (µg/µl)	protein amount (µg/ml)
pCO 117	0.993	19.92	1.992	1992
pQE 30-choA	0.748	14.94	1.494	1494

3. Calculation of enzyme activity

Temperature	A_{500} nm	A_{500} nm control	A observed-A control	Enzyme activity (U/ml)	Protein (µg/ml)	Specific activity (mU/mg protein)
At 37°C						
pCO 117	1.603	0.007	1.596	0.941	1992	472.6
pQE 30-choA	0.079	0.007	0.072	0.042	901.0	470.2
At 22°C						
pCO 117	0.746	0.000	0.745	0.440	1992	220.8
pQE 30-choA	0.026	0.000	0.026	0.015	550.6	279.2

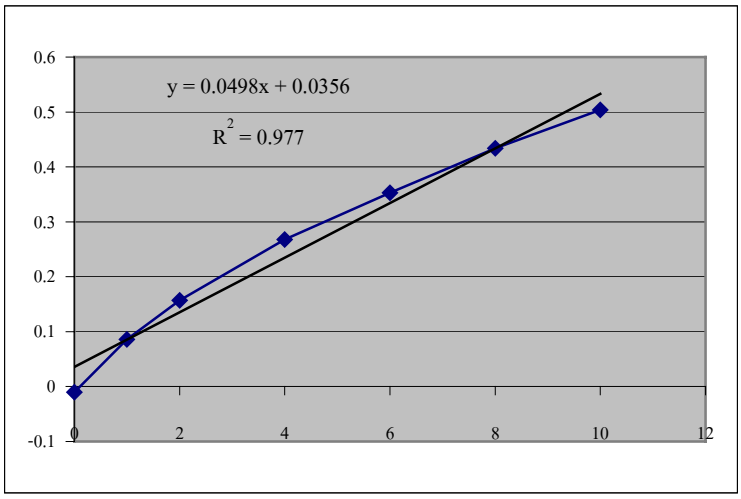
This enzyme activity was conducted by using Allain's *et al*. (1974) method at 37°C and 22°C.

APPENDIX H

IPTG concentration

1. BSA standard curve

BSA concentration (µg/(µl)	Absorbance 595 nm
0	-0.010
1	0.086
2	0.157
4	0.268
6	0.353
8	0.434
10	0.504



2. Calculation of protein concentration

IPTG (mM)	Time (h)	A_{595} nm	X Value (ug/30 ul)	X/30 (ug/ul)	protein amount (ug/ml)
Before induce	0	0.266	4.626	0.154	154.21
IPTG 0.1	1	0.312	5.550	0.555	555.0
	2	0.294	5.188	0.518	518.8
	3	0.294	5.188	0.518	518.8
	4	0.379	6.895	0.689	689.5
	5	0.424	7.799	0.779	779.9
	6	0.498	9.285	0.928	928.5
	8	0.535	10.028	0.334	334.2
	10	0.567	10.670	0.355	355.6
	18	0.501	9.345	0.311	311.5
	24	0.477	8.863	0.295	295.4
IPTG 0.5	1	0.294	5.188	0.173	172.9
	2	0.283	4.967	0.165	165.5
	3	0.359	6.494	0.216	216.4
	4	0.345	6.212	0.207	207.0
	5	0.43	7.919	0.264	263.9
	6	0.398	7.277	0.242	242.5
	8	0.487	9.064	0.302	302.1
	10	0.532	9.967	0.332	332.2
	18	0.491	9.144	0.304	304.8
	24	0.456	8.441	0.281	281.3

2. Calculation of protein concentration (continued)

IPTG (mM)	Time (h)	A_{595} nm	X Value (ug/30 ul)	X/30 (ug/ul)	protein amount (ug/ml)
IPTG 1.0	1	0.29	5.108	0.170	170.2
	2	0.278	4.867	0.162	162.2
	3	0.335	6.012	0.200	200.4
	4	0.311	5.530	0.184	184.3
	5	0.319	5.690	0.189	189.6
	6	0.388	7.076	0.235	235.8
	8	0.429	7.899	0.263	263.3
	10	0.548	10.282	0.343	342.9
	18	0.504	9.405	0.313	313.5
	24	0.471	8.743	0.291	291.4
IPTG 1.5	1	0.275	4.807	0.1602	160.2410
	2	0.300	5.309	0.1770	176.9746
	3	0.310	5.510	0.1837	183.6680
	4	0.368	6.674	0.2225	222.4900
	5	0.357	6.453	0.2151	215.1272
	6	0.407	7.457	0.2486	248.5944
	8	0.447	8.261	0.2754	275.3681
	10	0.505	9.425	0.3142	314.1901
	18	0.49	9.124	0.3041	304.1499
	24	0.451	8.341	0.2780	278.0455

2. Calculation of protein concentration (continued)

IPTG (mM)	Time (h)	A_{595} nm	X Value (ug/30 ul)	X/30 (ug/ul)	protein amount (ug/ml)
IPTG 2	1	0.287	5.048	0.1683	168.2731
	2	0.309	5.490	0.1830	182.9987
	3	0.312	5.550	0.1850	185.0067
	4	0.353	6.373	0.2124	212.4498
	5	0.377	6.855	0.2285	228.5141
	6	0.419	7.698	0.2566	256.6265
	8	0.46	8.522	0.2841	284.0696
	10	0.531	9.947	0.3316	331.5930
	18	0.504	9.405	0.3135	313.5207
	24	0.476	8.843	0.2948	294.7791
IPTG 2.5	1	0.309	5.490	0.183	182.9
	2	0.301	5.329	0.177	177.6
	3	0.335	6.012	0.200	200.4
	4	0.373	6.775	0.225	225.8
	5	0.427	7.859	0.262	261.9
	6	0.454	8.401	0.280	280.0
	8	0.514	9.606	0.320	320.2
	10	0.51	9.526	0.317	317.5
	18	0.508	9.485	0.316	316.1
	24	0.463	8.582	0.286	286.0

3. Calculation of enzyme specific activity

IPTG (mM)	Time (h)	A_{500} nm	A_{500} nm control	A observed-A control	Enzyme activity (U/ml)	Protein (μg/ml)	Specific activity (mU/mg protein)
Before induction	0	0.031	0.001	0.030	0.017	154.2169	0.114
IPTG 0.1	1	0.272	0.001	0.271	0.159	555.0	0.288
	2	0.264	0.001	0.263	0.155	518.8	0.299
	3	0.271	0.001	0.27	0.159	518.8	0.307
	4	0.295	0.001	0.294	0.173	689.5	0.251
	5	0.293	0.001	0.292	0.172	779.9	0.220
	6	0.305	0.001	0.304	0.179	928.5	0.193
	8	0.286	0.001	0.285	0.168	334.2	0.503
	10	0.241	0.001	0.24	0.141	355.6	0.398
	18	0.063	0.001	0.062	0.036	311.5	0.117
	24	0.018	0.001	0.017	0.010	295.4	0.033
IPTG 0.5	1	0.031	0.001	0.03	0.017	172.9	0.102
	2	0.216	0.001	0.215	0.126	165.5	0.766
	3	0.311	0.001	0.31	0.182	216.4	0.844
	4	0.281	0.001	0.28	0.165	207.0	0.797
	5	0.298	0.001	0.297	0.175	263.9	0.663
	6	0.247	0.001	0.246	0.145	242.5	0.598
	8	0.251	0.001	0.25	0.147	302.1	0.488
	10	0.245	0.001	0.244	0.144	332.2	0.433
	18	0.102	0.001	0.101	0.059	304.8	0.195
	24	0.023	0.001	0.022	0.013	281.3	0.046

3. Calculation of enzyme specific activity (continued)

IPTG (mM)	Time (h)	A_{500} nm	A_{500} nm control	A observed-A control	Enzyme activity (U/ml)	Protein (μg/ml)	Specific activity (mU/mg protein)
IPTG 1.0	1	0.220	0.001	0.219	0.129	170.2	0.758
	2	0.233	0.001	0.232	0.136	162.2	0.843
	3	0.274	0.001	0.273	0.161	200.4	0.803
	4	0.259	0.001	0.258	0.152	184.3	0.825
	5	0.246	0.001	0.245	0.144	189.6	0.762
	6	0.235	0.001	0.234	0.138	235.8	0.585
	8	0.304	0.001	0.303	0.178	263.3	0.678
	10	0.255	0.001	0.254	0.149	342.9	0.436
	18	0.125	0.001	0.124	0.073	313.5	0.233
	24	0.036	0.001	0.035	0.020	291.4	0.070
IPTG 1.5	1	0.268	0.001	0.267	0.157	160.2	0.983
	2	0.276	0.001	0.275	0.162	176.9	0.916
	3	0.294	0.001	0.293	0.172	183.6	0.941
	4	0.275	0.001	0.274	0.161	222.4	0.726
	5	0.288	0.001	0.287	0.169	215.1	0.787
	6	0.29	0.001	0.289	0.170	248.5	0.685
	8	0.288	0.001	0.287	0.169	275.3	0.614
	10	0.258	0.001	0.257	0.151	314.1	0.482
	18	0.133	0.001	0.132	0.077	304.1	0.256
	24	0.036	0.001	0.035	0.020	278.0	0.074

3. Calculation of enzyme specific activity (continued)

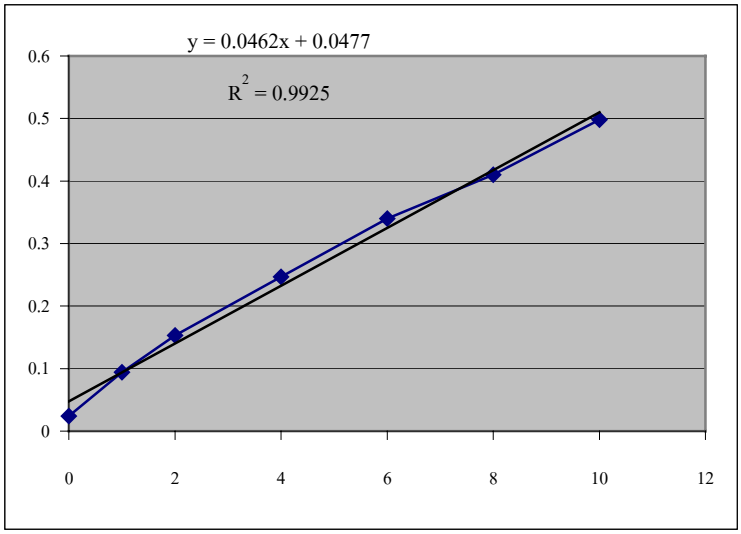
IPTG (mM)	Time (h)	A_{500} nm	A_{500} nm control	A observed-A control	Enzyme activity (U/ml)	Protein (μg/ml)	Specific activity (mU/mg protein)
IPTG 2	1	0.235	0.001	0.234	0.138	168.2	0.820
	2	0.269	0.001	0.268	0.158	182.9	0.864
	3	0.263	0.001	0.262	0.154	185.0	0.835
	4	0.274	0.001	0.273	0.161	212.4	0.758
	5	0.292	0.001	0.291	0.171	228.5	0.751
	6	0.28	0.001	0.279	0.164	256.6	0.641
	8	0.257	0.001	0.256	0.151	284.0	0.531
	10	0.274	0.001	0.273	0.161	331.5	0.485
	18	0.181	0.001	0.18	0.106	313.5	0.338
	24	0.054	0.001	0.053	0.031	294.7	0.106
IPTG 2.5	1	0.25	0.001	0.249	0.146	182.9	0.802
	2	0.244	0.001	0.243	0.143	177.6	0.807
	3	0.271	0.001	0.27	0.159	200.4	0.794
	4	0.273	0.001	0.272	0.160	225.8	0.710
	5	0.279	0.001	0.278	0.164	261.9	0.626
	6	0.24	0.001	0.239	0.141	280.0	0.503
	8	0.26	0.001	0.259	0.152	320.2	0.477
	10	0.216	0.001	0.215	0.126	317.5	0.399
	18	0.094	0.001	0.093	0.054	316.1	0.173
	24	0.032	0.001	0.031	0.018	286.0	0.063

APPENDIX I

Density of inoculum

1. BSA standard curve

BSA concentration (µg/(µl)	A_{595} nm
0	0.024
1	0.094
2	0.153
4	0.247
6	0.340
8	0.410
10	0.498



2. Calculation of protein concentration

Density of inoculum (ml)	Time (h)	A_{595} nm	X Value (μg/30 ul)	X/30 (μg/μl)	protein amount (mg/ml)
0.25 ml	0	0.210	3.502	0.116	116.7
	1	0.265	4.606	0.153	153.5
	2	0.289	5.088	0.169	169.6
	3	0.313	5.570	0.185	185.6
	4	0.326	5.831	0.194	194.3
	6	0.408	7.477	0.249	249.2
	19	0.438	8.080	0.269	269.3
1 ml	0	0.237	4.044	0.134	134.8
	1	0.292	5.148	0.171	171.6
	2	0.325	5.811	0.193	193.7
	3	0.273	4.767	0.158	158.9
	4	0.336	6.032	0.201	201.1
	6	0.417	7.658	0.255	255.2
	20	0.395	7.216	0.240	240.5
2 ml	0	0.204	3.381	0.112	112.7
	1	0.244	4.184	0.139	139.4
	2	0.364	6.594	0.219	219.8
	3	0.328	5.871	0.195	195.7
	4	0.289	5.088	0.169	169.6
	6	0.438	8.080	0.269	269.3
	21	0.457	8.461	0.282	282.1

2. Calculation of protein concentration (continued)

Density of inoculum (ml)	Time (h)	A_{595} nm	X Value (μg/30 ul)	X/30 (μg/μl)	protein amount (mg/ml)
4 ml	0	0.214	3.582	0.119	119.4
	1	0.243	4.164	0.138	138.8
	2	0.270	4.706	0.156	156.8
	3	0.350	6.313	0.210	210.4
	4	0.353	6.373	0.212	212.4
	6	0.383	6.975	0.232	232.5
	10	0.475	8.823	0.294	294.1
	22	0.429	7.899	0.263	263.3

2. Calculation of enzyme activity

Density of inoculum (ml)	Time (h)	A_{500} nm	A_{500} nm control	A observed-A control	Enzyme activity (U/ml)	Protein (μg/ml)	Specific activity (mU/mg protein)
0.25 ml	0	0.046	0.001	0.045	0.026	116.7	0.227
	1	0.142	0.001	0.141	0.083	153.5	0.541
	2	0.207	0.001	0.206	0.121	169.6	0.716
	3	0.241	0.001	0.24	0.141	185.6	0.762
	4	0.278	0.001	0.277	0.163	194.3	0.840
	6	0.281	0.001	0.28	0.165	249.2	0.662
	19	0.317	0.001	0.316	0.186	269.3	0.6922

3. Calculation of enzyme activity (continued)

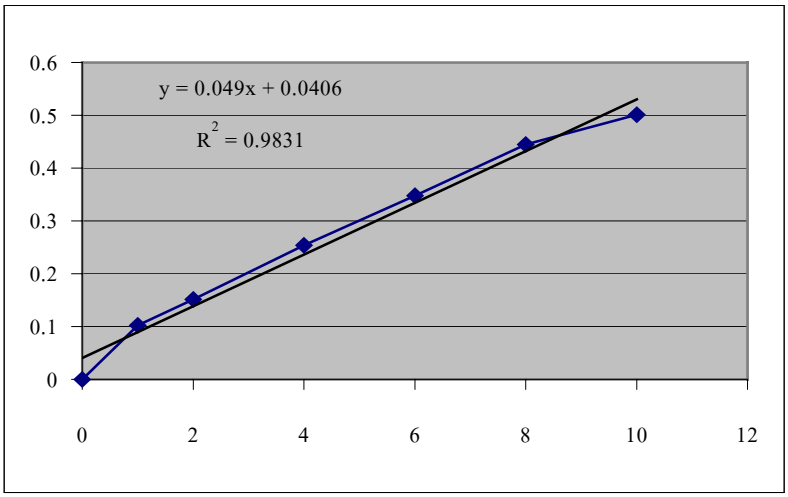
Density of inoculum (ml)	Time (h)	A_{500} nm	A_{500} nm control	A observed- A control	Enzyme activity (U/ml)	Protein (μg/ml)	Specific activity (mU/mg protein)
1 ml	0	0.057	0.001	0.056	0.033	134.8059	0.245
	1	0.19	0.001	0.189	0.111	171.6198	0.649
	2	0.253	0.001	0.252	0.148	193.7082	0.767
	3	0.304	0.001	0.303	0.178	158.9023	1.125
	4	0.289	0.001	0.288	0.169	201.071	0.845
	6	0.378	0.001	0.377	0.222	255.2878	0.871
	20	0.269	0.001	0.268	0.158	240.5622	0.657
2 ml	0	0.066	0.001	0.065	0.038	112.7175	0.340
	1	0.164	0.001	0.163	0.096	139.4913	0.689
	2	0.314	0.001	0.313	0.184	219.8126	0.840
	3	0.289	0.001	0.288	0.169	195.7162	0.868
	4	0.383	0.001	0.382	0.225	169.6118	1.328
	6	0.402	0.001	0.401	0.236	269.344	0.878
	21	0.296	0.001	0.295	0.174	282.0616	0.617
4 ml	0	0.113	0.001	0.112	0.066	119.411	0.553
	1	0.224	0.001	0.223	0.131	138.822	0.947
	2	0.35	0.001	0.349	0.205	156.8942	1.312
	3	0.347	0.001	0.346	0.204	210.4418	0.970
	4	0.443	0.001	0.442	0.260	212.4896	1.227
	6	0.489	0.001	0.488	0.287	232.5301	1.238
	10	0.482	0.001	0.481	0.283	294.1098	0.964
	22	0.452	0.001	0.451	0.266	263.3199	1.010

APPENDIX J

Assay method

1. BSA standard curve

BSA concentration (µg/(µl)	A_{595} nm
0	0.000
1	0.102
2	0.151
4	0.253
6	0.347
8	0.445
10	0.501
20	0.761



2 Calculation of protein concentration

Sample	A_{595} nm	X Value (µg/10 ul)	X/10 (µg/µl)	protein amount (µg/ml)
1	0.059	0.385	0.038	38.57
2	0.069	0.579	0.058	57.95

3. Calculation of enzyme activity

Method	A_{500} nm	A_{500} nm control	A observed-A control	Enzyme activity (U/ml)	Protein (µg/ml)	Specific activity (mU/mg protein)
Masurekar and Goodhue (1978)						
1	0.103	0	0.103	0.061	38.57	158.62
2	0.115	0	0.115	0.067	57.95	117.17
Allain *et al.* (1974)						
1	0.384	-0.001	0.385	0.227	38.57	589.67
2	0.455	-0.001	0.456	0.269	57.95	464.39

BIBLIOGRAPHY

Name : Potjamarn Suraninpong

Date of Birth : April 30, 1968 Place of Birth : Narathiwat

Nationality : Thai Religion : Buddhism

Permanent address : 19/13 Moo 5, Su-Ngai-Go-Log District, Narathiwat, 96120.

Working address : Institute of Agricultural Technology, Walailak University, 222 Thai Buri Sub-district, Tha Sala District, Nakhon Si Thammarat, 80160.

Education

Year	Degree	Institution	Major
1987-1991	Bachelor of Science (Agriculture)	Kasetsart University	Agronomy
1992-1995	Master of Science (Agriculture) (Scholarship : NSTDA)	Prince of Songkla University	Plant Science
1998-2002	Doctor of Philosophy	Suranaree University of Technology	Crop Production Technology

(Scholarship : Royal Golden Jubilee, The Thailand Research Fund)

Work Experiences

Year	Position	Employer
1996-1997	Co-op Coordinator	Suranaree University of Technology
1997-1998	Lecturer	Prince of Songkla University
2002-Present	Lecturer	Walailak University

Education Experiences

12-30 March 1990	Practice on research and development of corn and sorghum processing and marketing at GIBA-GEIGY, Nakhon Sawan, Thailand.
4 Oct - 2 Nov 1990	Short-term farm practice program at Tokyo University of Agriculture,Japan.
21-25 Dec 1998	Attended the workshop on the application of molecular markers vegetable and crop production : Advances in plant breeding methodologies, Kasetsart University, Thailand.
6-8 Oct 1999	Attended the 11st National Genetic Conference at Suranaree University of Technology, Thailand.
18-20 Jan 2000	Attended the 8th Mungbean Meeting at Kasetsart University, Thailand.
25-27 Jan 2000	Attended the workshop on gene transfer at Suranaree University of Technology, Thailand.
12 Sept 2000 – 30 Apr 2001	Visiting research scholar at Dept. of Crop Sciences, University of Illinois at Urbrana-Champaing, USA for Ph.D. dissertation sponsored by The Thailand Research Fund (Royal Golden Jubilee Program).

Research Papers

1. Te-chato, S., M. Lim, M. Chumroenruk and **P. Suraninpong**. 1994. Effect of phlorogucinol on root induction of mangosteen (*Garcinia mangostana* L.) *in vitro*. Khon Kaen Agic. 22 (3) : 127-132. (in Thai)
2. Te-chato, S., **P. Suraninpong** and S. Chuenjit. 1995. Identification of rubber callus resistant to culture filtrate of Phytophthora spp. Songkhlanakarin J. Sci. Technol. 17(1) : 1-6.
3. Te-chato, S., **P. Suraninpong** and S. Chuenjit. 1995. Screening of rubber callus resistance to phytophthora leaf fall agent. Songkhlanakarin J. Sci. Technol. 17(1) : 7-15.

4. Te-chato, S., M. Lim and **P. Suraninpong**. 1995. Embryogenic callus induction in mangosteen (*Garcinia mangostana* L.) Songkhlanakarin J. Sci. Technol. 17(2) : 115-120.
5. Te-chato, S., M. Lim and **P. Suraninpong**. 1995. Type of medium and cytokinins in relation with purple leaf and callus formation of mangosteen. Songkhlanakarin J. Sci. Technol. 17(2) : 129-135.
6. Te-chato, S., M. Lim and **P. Suraninpong**. 1995. Plantlet formation from leaf-derived embryogenic callus of mangosteen. Songkhlaanakarin J. Sci. Technol. 17(2) : 115-120.
7. **Suraninpong P.** and Te-chato, S. 1998. Root induction from in vitro regenerated shoot of somkaek (*Garcinia atroviridis*). Khon Kaen Agic. 26 (2) : 74-84. (in Thai)
8. **Suraninpong P.** and Te-chato, S. 1998. *In vitro* propagation of *Oxalis corymbosa* D.C. Journal of Agriculture 14 (1) : 87-98. (in Thai)
9. **Suraninpong P.** and Te-chato, S. 1999. Effect of cytokinins on cell suspension culture, isolation and culture of protoplast of rubber. Songkhlanakarin J. Sci. Technol. 21(2) : 169-177. (in Thai)
10. **Suraninpong P.**, Chanyuth, S. and Waranyuwat, A. 2000. Sugarcane improvement for salt tolerance. Suranaree J. Sci. Technol. 7:217-223. (in Thai)

Presentation

1. **Suraninpong, P**., S. te-chato, and S. Chuenjit. Preliminary screening of rubber calli with culture fittrate of *Phytophthora palmivora* Butler. Poster showed at USU-USM-PSU Scientific Seminar in IMT Triangle Zone at Medan, Indonesia, 28 May-3 June 1994.

2. **Suraninpong, P**., S. te-chato, and S. Chuenjit. Selection of rubber (*Hevea brasiliensis* Muell. Arg.) callus for resistance to a culture filtrate of phytophthora leaf fall agents. Oral presentation in The Conference on Science, Technology and Environment for Rural Development at Kasetsart University Thailand, 21-23 December 1994.

3. **Suraninpong, P**., Sontichai Chanprame, Hyeon-Je Cho, Jack M. Widholm and Aree Waranyuwat. An expression of a cholesterol oxidase gene in mungbean (*Vigna radiata*)using *Agrobacterium rhizogenes*-mediated transformation. Poster showed in RGJ Seminar Series : AggieNet I "plant Science" at Kasetsart University, Thailand, 25 September 2001.
4. **Suraninpong, P**., Sontichai Chanprame, Hyeon-Je Cho, Jack M. Widholm and Aree Waranyuwat. *Agrobacterium*-mediated transformation system in mungbean [*Vigna radiata* (L.) Wilczek]. Oral presentation in RGJ-Ph.D. Congress III at Chomteian Palm Beach Resort, Chonburi, Thailand, 25-27 April 2002.
5. **Suraninpong, P**., Sontichai Chanprame, Hyeon-Je Cho, Jack M. Widholm and Aree Waranyuwat. Cloning and expression of cholesterol oxidase gene in a bacterium *E. coli* M15 (pREP4). Oral presentation in The 14th Annual Meeting of the Thai Society for Biotechnology : Biotechnology for Better Living in the New Economy at Hotel Sofitel Raja Orchid, Khon Kaen, Thailand, 12-15 November 2002.