การแสดงออกของ *TGG1* โปรโมเตอร์ ในพืชวงศ์ Solanaceae บางชนิด

นางสาวรักชนก โคโต

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EXPRESSION OF *TGG1* PROMOTER IN SOME SOLANACEOUS PLANT SPECIES

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ยีน *TGG* มีบทบาทในการป้องกันตัวเองของพืช ยีน *gus* ที่ถูกควบคุมการแสดงออกด้วยโปรโมเตอร์ของยีน*TGG1* จาก *Arabidopsis* (*Arabidopsis thaliana* L.) ซึ่งเป็นยีนที่มีการแสดงออกอย่างจำเพาะต่อเซลล์คุมของ *Arabidopsis* ถูกถ่ายเข้าสู่ยาสูบ พิทูเนีย และ มันฝรั่ง ซึ่งเป็นพืชในวงศ์ Solanaceae และไม่มีระบบการทำงานของ เอนไซม์ TGG โดย *Agrobacterium tumefaciens* LBA 4404 ProA1.1 ได้ต้นยาสูบ พิทูเนีย และมันฝรั่งที่ถูกถ่ายยีนแล้ว 10 3 และ 9 สายพันธุ์ ตามลำดับ การแสดงออกอย่างจำเพาะของ ยีน *gus* ที่เซลล์คุมพบบริเวณใบเลี้ยง ใบแท้ ก้านดอก กลีบเลี้ยง กลีบดอก อับเรณู เรณู และก้านชูยอดเกสรตัวเมียในต้นยาสูบ และในใบแท้ กลีบเลี้ยง กลีบดอก อับเรณูในพิทูเนียที่ได้รับการถ่ายยีนดังกล่าว ส่วนใบแท้และลำต้นของมันฝรั่งที่ถูกถ่ายยีนไม่พบตำแหน่งการแสดงออกของยีน *gus* ที่เซลล์คุม เมื่อทำการศึกษาการแสดงออกของ ยีน*gus* เชิงปริมาณพบว่าใบเลี้ยงของยาสูบอายุ 15 วัน ที่ได้รับการถ่ายยีนแล้วมีการแสดงออกสูงที่สุด สำหรับพิทูเนียที่ได้รับการถ่ายยีน เกสรตัวเมียเป็นส่วนที่มีการแสดงออกสูงที่สุด ส่วนต้นมันฝรั่งที่ได้รับการถ่ายยีนแล้วทุกสายพันธุ์มีค่า GUS activity ต่ำ เมื่อตรวจวัดโดยวิธีทางฟลูออเรสเซนต์ แต่เมื่อตรวจสอบโดยวิธีทางฮิสโตเคมิคัลแล้ว ไม่พบ GUS activity เนื้อเยื่ออ่อนทั้ง ในอวัยวะสืบพันธุ์และอวัยวะอื่นๆของยาสูบและพิทูเนียที่ถูกถ่ายยีนแล้วมีการแสดงออกสูงกว่าเนื้อเยื่อแก่ และพบว่ายาสูบที่ถูกถ่ายยีนแล้วที่ถูกเพาะเลี้ยงในขวดทดลองมีปริมาณการแสดงออกของยีน *gus* ที่สูงกว่าต้นที่ปลูกในดิน ผลการทดลองในครั้งนี้แสดงให้เห็นว่า ชนิดพืช อายุของเนื้อเยื่อ และสิ่งแวดล้อม มีผลต่อการแสดงออกของ โปรโมเตอร์ของ *Arabidopsis TGG1*

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สาขาวิชา พันธุศาสตร์ ..... ลายมือชื่ออาจารย์ที่ปรึกษา.....
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Abstract : *Gus* gene regulated by *Arabidopsis TGG1* promoter, showing the specific expression in *Arabidopsis* (*Arabidopsis thaliana*) guard cells, was transformed into tobacco (*Nicotiana tabacum* L.), petunia (*Petunia hybrida* L.) and potato (*Solanum tuberosum* L.), the Solanaceous plant species containing no TGG enzyme system, by *Agrobacterium tumefaciens* LBA 4404 ProA1.1. Ten independent transgenic tobacco lines, three transgenic petunia lines and nine transgenic potato lines were obtained. The guard cell specific expression was detected in cotyledons, leaves, pedicels, sepals, petals, anthers, and styles of the transgenic tobacco and in leaves, sepals, petals, anthers of transgenic petunia. No guard cell specific expression was found in leaves and stems of transgenic potato. The quantitative analysis of *gus* gene expression indicated the highest GUS activity in cotyledons of 15-day-after germination T_2 transgenic tobacco plant. For the transgenic petunia, the highest GUS activity was detected in pistils. Low level of GUS activity in leaves and stems of all transgenic potato lines was fluorometrically detected, but it could not be detected histochemically. The younger tissues had the higher level of GUS activity than the older ones in both vegetative and reproductive organs. The aseptically grown transgenic tobacco had the higher level of GUS activity than the soil grown transgenic ones. These data suggest the species, age and environmental effect on *Arabidopsis TGG1* promoter expression.

Department Botany ……Student's signature……...

Field of study Genetics ……..Advisor's signature……...

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CHAPTER I

INTRODUCTION

Thioglucoside glucohydrolase, E.C.3.2.3.1(TGG), commonly referred to as myrosinase, catalases the hydrolysis of glucosinolate (GS), a group of sulfur-containing glucosides present in all crucifers (Fenwick et al.,1983). The normal products of the enzymatic degradation are glucose and isothiocyanate (Larsen, 1981). However, other products; nitrides, thiocyanates, amines, thiones, cyanoepithioalkanes, can be produced, depending on pH and substrates. These physiologically active products not only contribute to the distinct flavor and aroma characteristics of crucifers, but also have undesirable effects on feeding animals due to their pungency and goitrogenic activity (Larsen,1981;Fenwick and Heaney, 1983). Moreover, isothiocyanates are considered to be antimicrobial to plant pathogen (Walker *et al*., 1937; Beizile *et al*., 1962; Drobnica *et al*., 1967; Holly and Jones, 1985) and affect insect herbivore behavior which are self defense in plant (Hicks, 1974; Blau *et al*, 1978; Renwick and Radke, 1987).

Molecular studies of *TGG* gene families have been pursued in many Crucifers. The *TGG* gene family in Arabidopsis is composed of 3 genes; *TGG1*, *TGG2*, and *TGG3*. *TGG1* and *TGG2* are expressed in all green tissues, while *TGG3* is probably a silent pseudogene. The *TGG1* and *TGG2* genes are both expressed in the same tissues; leaf, cauline leaf, bract, inflorescence stem, flower bud, flower, and silique. Based on results with *TGG1* promoter-*gus* gene expression, *TGG1* is also expressed in developing seeds and imbibe seeds. However, neither *TGG1* nor *TGG2* mRNA transcripts could be detected in seed tissues. This may be because the steady state levels of *TGG1* and *TGG2* mRNA are too low to be detected by a northern blot hybridization experiment.

TGG1 promoter-*gus* gene expression in transgenic *Arabidopsis* shows localization in guard cells in all examined tissues; cotyledon, hypocotyl, leaf, bract, cauline leaf, inflorescence stem, sepal, anther, style, and silique. The guard cell specific expression of the *TGG* genes was confirmed with *in situ* hybridization, using *TGG1* and *TGG2* gene specific probes (Chadchawan,1995).

The *TGG* gene family in *Arabidopsis* is the first gene family whose expression has been demonstrated to be localized uniquely in guard cells and has a function in the plant self defense system. The first report of guard cell localization of gene expression was the *rha* 1 gene in *Arabidopsis* (Terry *et al*, 1993), which encodes a small GTP binding protein. Significant *rha* 1 expression was also found in stipules, root tips of young plants, and receptacles of flowers, and therefore is not an example of tight guard cell specific expression. The other gene, which also shows expression in guard cells, is *ADP-glucose pyrophorelase* from potato (Muller-Rober *et al*, 1994). The *AGPase* gene is active in various starch containing cells, including guard cells, tuber parenchyma cells, and starch sheath layer of stem and petiole. Muller-Rober *et al*. (1994) identified a 0.3 kb 5' proximal promoter fragment as being sufficient to obtain exclusive expression in guard cells of transgenic potato and tobacco plants. Comparing the *TGG1* gene with the other two genes showing expression in guard cells, the *TGG1* gene has a much more restricted cell type-specific expression pattern than the other guard cell expressed genes.

It will be interesting to know if the *TGG1* promoter exhibits the guard cell-specific expression pattern in other myrosinase containing species, and in species that do not have myrosinase containg cells (myrosin cells). The latter question can be answered by transforming the *TGG1* promoter-*gus* gene fusion construct into plant species containing no TGG activity or myrosin cells, such as some Solanaceous plant species. Then *gus* gene expression would be determined in those transgenic plants.

The histology of transgenic tissues stained for GUS enzyme activity will tell us if the *TGG1* promoter can function in plant species containing no myrosin cells and, if can, which cell types contain the product. The cellular and the level pattern of *TGG1* promoter expression could also be conveniently determined by histochemical and fluorometric assay, respectively.

The objective of this research was :

Study the expression of *TGG1* promoter-*gus* gene in transgenic Solanaceous plants

The strategies to be used were :

1. Selection of the Solanaceous plant species to be used as the representatives for plants containing no TGG-glucosinolate system ; i.e., tobacco (*Nicotiana tabaccum*
   L.), petunia (*Petunia hybrida* L.), or potato (*Solanum tuberosum* L.).
2. Preparation of aseptically cultured plant material.
3. Transformation of *TGG1* promoter-*gus* gene (via *Agrobacterium tumefaciens* LBA4404) into selected plants species.
4. Selection of the transformed plants by using kanamycin resistance as a selectable marker.
5. Study of *TGG1* promoter-*gus* gene expression in transgenic plants by GUS histochemical and fluorometric assay.

CHAPTER II

LITERATURE REVIEW

I.*TGG1* promoter

1. Glucosinolates (GS)

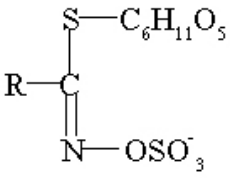
Glucosinolates occur in important crop plants and vegetables, mainly from the family Cruciferae (Larsen, 1981). Bones and Iversen (1985) indicated that they were found in many families in the Order Capparales, specifically in members of the Family Capparidaceae, Cruciferae, Resedaceae, in which glucosinolates seem to be present in all species (Larsen, 1981).

Glucosinolates are found in all parts of the plants and up to 15 different glucosinolates have been found in the same plant (Larsen, 1981; Sang *et al*, 1984). Ettlinger and Lundeen (1956) proposed the correct structural formula for sinigrin (allyglucosinolate) and sinalbin (p-hydroxybenzylglucosinolate), based on the chemical studies of both glucosinolates (Figure 1). The glucosinolate skeleton consists of a thioglucosidic link to the carbon of a sulphonated oxime which is derived from amino acids. The amino acid derived part of the structure (R-group) is variable and can be aliphatic (e.g. alkenyl, hydroxyalkenyl, W-methylthioalkyl), aromatic (e.g. benzyl, substituted benzyl) or heterocyclic (e.g. indolyl).

2. Degradation of glucosinolates

All plants containing glucosinolates (GS) contain enzymes generally called myrosinases (Thioglucoside glucohydrolase enzymes ; TGG) which hydrolyze the GS. The normal products of the enzymatic degradation are glucose and isothiocyanate (Larsen, 1981). However, other products; nitrides, thiocyanates, amines, thiones, cyanoepithioalkanes, can be produced, depending on pH and substrates (Figure 2) . These physiologically active products not only contribute to the distinct flavor and aroma characteristics of crucifers, but also have undesirable effects on feeding animals due to their pungency and goitrogenic activity (Larsen, 1981; Fenwick and Heaney, 1983). Moreover, isothiocyanates are considered to be antimicrobial (Walker *et al*., 1937; Beizile *et al*., 1962; Drobnica *et al*., 1967; Holly and Jones, 1985) and affect insect herbivore behavior (Hicks, 1974; Blau *et al*, 1978; Renwick and Radke, 1987).

Isothiocyanates are one of glucosinolate hydrolysis products with the most characterized antimicrobial activity. This group of substances is toxic to a wide range of fungi (Drobnica *et al*., 1967; Greenhalgh and Mitchell, 1976; Mithen *et al*., 1986), including Nematospora yeast (*Nematospora coryli*), which infects some *Brassica* species (Holley and Jones, 1984). Indole products of glucosinotate hydrolysis also inhibit the growth of *Leptoshria maculans* (Blackleg) in culture (Mithen *et al*, 1986). Mithen *et al*. (1987) showed that levels of alkenyl glucosinolate in leaves of *Brassica napus* and its two putative parents, *B. rapa* and *B. oleracea*, were inversely correlated with lesion extension as caused by *L. maculans*. However, there was no clear association between hypersensitive resistance and glucosinolate content.



The general formular of glucosinolate

R = CH_2=CH-CH_2 Sinigrin (allylglusinolate)

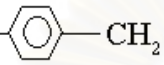
R = HO—  —CH_2 Sinalbin (p-hydroxybenzylglucosinolate)

Figure 1.The structural formular of glucosinolates proposed by Ettlinger and Lundeen (1956).

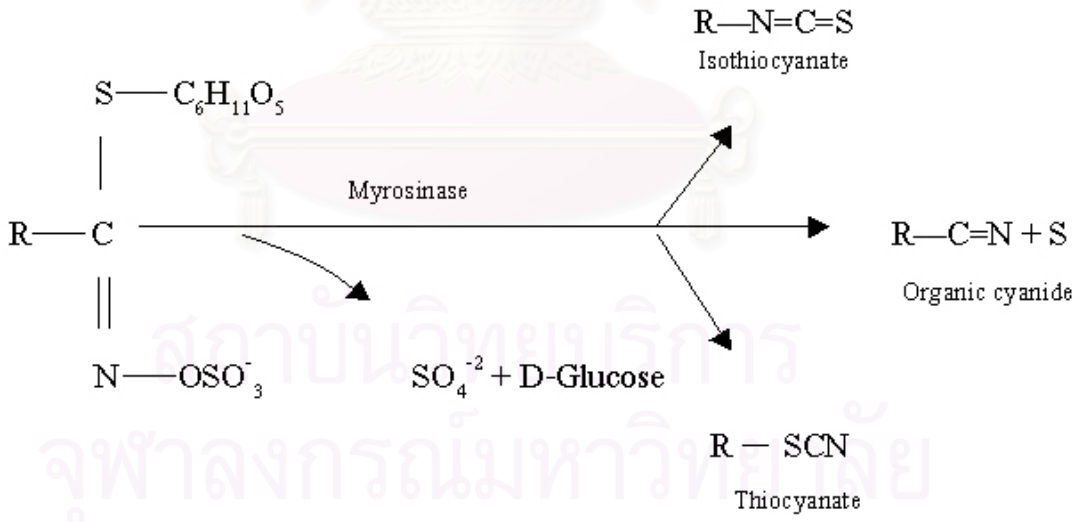


Figure 2 Degradation reaction of glucosinolates catalysed by TGG (myrosinase) enzyme.

Peronospora parasitica (downy mildew) is another *Brassica* fungal pathogen whose pathenogenogenicity was inversely correlated with the amount of allylisothiocyanate concentration in plant seedlings (Greenhalgh and Dickinson, 1976; Greenhalgh and Mitchell, 1976). In addition, downy mildew resistance of wild *Brassica* populations was correlated with a high concentration of isothiocynates. They also performed on *in vitro* study showing that allylisothiocyanate was remarkably active in inhibition of sporangium germination of *Peronospora parasitica* , while allyl nitrite had no effects on sporangium germination (Greenhalgh and Dickinson,1976; Greenhalgh and Mitchell, 1976).

However, some GSs still have toxic effects to some other insects which do not normally attack Crucifers (Blau *et al*, 1978). Allyl glucosinolates have to be acutely toxic to *Papilio polyvenes* larvae, which do not normally attack Crucifers. High concentration of allyl glucosinolates was also toxic to a generalist feeder, *Spodopteraeridania*. By contrast, larva growth of *Pierisrapae*, a Crucifer feeder, was not affected by high concurrence with Hicks's study (1974), showing that sinigrin was not toxic to a Crucifer feeder, but that sinigrin acted as a stimulant to flea beetles (Phyllotretacruciferae). It was suggested that this was insect adaptation to the toxic effects of glucosinolate hydrolysed products. He called it " a turn of the evolutionary scale" in which these insect pests not only developed tolerance to the potential toxins but have made use of them and their glucosides for finding their food-plants more efficiently.

The TGG enzymes are sepearated from their substrates in the intact plant tissues. Glucosinolate are thought to localized to the vacuoles (Grob and Matile, 1979; Matile, 1980; Wei *et al*, 1981). TGGs are also sequestered in vacuoles (myrosin grains) but within specialized myrosin cells (Bones and Iversen, 1985). Thus, cellular disruption is necessary for glucosinolate hydrolysis to occur (Bjorkman, 1976). Therefore, events such as vegetable preparation (Mullin *et al*., 1980), for example,

cutting or chopping, or cell damage caused by pest or pathogen infection resulted in the accumulation of all the glucosinolate breakdown products.

3. Genes encoding TGG proteins

Molecular studies of *TGG* gene families have been pursued in many Crucifers. One full length cDNA clone and several partial cDNA clones containing *TGG* genes have been isolated and sequenced from *S. alba* (Xue *et al*, 1992). A genomic clone containing a myrosinase gene from *B. campestris* has been identified and the gene was sequenced (Machlin *et al*, 1993). Genomic Southern blots of *S. alba*, *B. napus* (Xue *et al*, 1992) and *B. campestris* (Machlin *et al*, unpublished data) showed that TGG proteins are encoded by gene families; approximately 16 gene in *B. napus* and 7 genes in *B. campestris*. Xue *et al* (1992) showed by Southern blot that *Arabidopsis* genomic DNA hybridized with a *S. alba* myrosinase probe and concluded that *Arabidopsis* had 3 *TGG* genes.

4. *TGG* genes in *Arabidopsis*

The *TGG* gene family in *Arabidopsis* is composed of 3 genes; *TGG1*, *TGG2*, and *TGG3*. *TGG1* and *TGG2* are expressed in all green tissues, while *TGG3* is probably a silent pseudogene. The *TGG1* and *TGG2* genes are both expressed in the same tissues; leaf, cauline leaf, bract, inflorescence stem, flower bud, flower, and silique. Based on results with *TGG1* promoter-*gus* gene expression, *TGG1* is also expressed in developing seeds and imbibe seeds. However, neither *TGG1* nor *TGG2* mRNA transcripts could be detected in seed tissues. This may be because the steady state levels of *TGG1* and *TGG2* mRNA are too low to be detected by a northern blot hybridization experiment.

TGG1 promoter-*gus* gene expression in transgenic *Arabidopsis* shows localization in guard cells in all examined tissue; cotyledon, hypocotyl, leaf, bract,

cauline leaf, inflorescence stem, sepal, anther, style, and silique. The guard cell specific expression of the *TGG* genes was confirmed with *in situ* hybridization, using *TGG1* and *TGG2* gene specific probes(Chadchawan,1995).

5. Guard cell specific promoter characterization

The *TGG* gene family in *Arabidopsis* is the first gene family whose expression has been demonstrated to be localized uniquely in guard cells and has a function in the plant self defense system. The first report of guard cell localization of gene expression was the *rha 1* gene in *Arabidopsis* (Terry *et al*, 1993), which encodes a small GTP binding protein. Significant *rha 1* expression was also found in stipules, root tips of young plants, and receptacles of flowers; and therefore it is not an example of tight guard cell specific expression. Another gene, which also shows expression in guard cells, is *ADP-glucose pyrophorelase* (*AGPase*) from potato (Muller-Rober *et al*, 1994). The *AGPase* gene is active in various starch containing cells, including guard cells, tuber parenchyma cells, and starch sheath layer of stem and petiole. Muller-Rober et al. (1994) identified a 0.3 kb 5' proximal promoter fragment as being sufficient to obtain exclusive expression in guard cells of transgenic potato and tobacco plants. CDE T6 – 19 of *Craterostigneum* (Taylor *et al*, 1995), which shows expression in guard cells, and nearly tissue of guard cell also expressed when treated with abscisic acid (ABA). *HBP* – 1a (17) promoter from *Arabidopsis*, which each promoter that also shows expression in guard cells, mesophyll and vascular bundles. Mikami *et al* (1995) found that *HBP* –1a (17) promoter contains several sequence elements homologous to *cis* – acting element of light – inducible promoter. Comparing the *TGG1* gene promoter with the other four genes showing expression in guard cells, the *TGG1* gene has a much more restricted, cell type-specific expression pattern than the other guard cell expressed genes.

II. *Agrobacterium*-mediated transformation

Efficient methods for introducing cloned genes into plants are important for understanding and controlling plant gene expression. The ability to manipulate genes could lead to rational, deliberate alternations of the crop plant genome for improvement of their agronomic performance. Production of morphological normal plants that contains the expression of foreign genes has been made possible by use of the natural gene-transfer capacity of *Agrobacterium tumefaciens* (Horsh *et al*, 1985).

The first record on transgenic tobacco plant expressing foreign genes appeared at beginning of the last two decades, although many of the molecular characteristics of process were unknow at that moment (Herrera-Estrella *et al*, 1983). Since that crucial moment in the development of plant science, a great progress in understanding the *A. tumefaciens* naturally infects only dicotyledonous plants and many economically important plants, including the cereals, remained accessible for genetic manipulation during long time. For these cases, alternative direct transformation methods have been developed (Shilito *et al*., 1985), such as polyethyleneglycol- mediated transfer (Uchimiya *et al*., 1986), microinjection (De la Pena *et al*., 1987), protoplast and intact cell electroporation (Lorz *et al*., 1985) and gene gun technology (Sanford, 1988). However, Agrobacterium-mediated transformation has remarkable advantages over direct transformation methods. It reduces the copy number of the transgene, potentially leading to fewer problems with transgene cosuppresion and instability (Hansen *et al*., 1997).

A.tumefaciens has the exceptional ability to transfer a paticular DNA segment (T – DNA) of the tumor – inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (Nester *et al*., 1984; Binns and Thomashaw, 1988). T- DNA contains two types of auxins and cytokinins, responsible for tumor formation, and the genes encoding for the synthesis of opines. These compounds, produced by

condensation between amino acids and sugars, are synthesized and excreted by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen sources. Outside the T – DNA, are located the genes for the opine catabolism, and the genes involved in bacterium-bacterium plasmid conjugative transfer. (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995).

Virulent strains of *A. tumefaciens*, when interacting with susceptible dicotyledonous plant cells, induce diseases known as crown gall. These strains contain a large megaplasmid (more thad 200kb) which play a key role in tumor induction and for this reason it was named Ti plasmid. Ti plasmids are classified according to the opines, which are produced and excreted by the tumors they induce. During infection the T – DNA, a mobile segment of Ti is transferred to the plant cell nucleus and integrated into the plant chromosome. The T – DNA fragment is flanked by 25 – bp direct repeats, which act as a cis element signal for the transfer apparatus. The process of T –DNA tranfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome. The Ti plasmid also contains the genes for opine catabolism produced by the crown gall cells, and regions for conjugative transfer and for its own integrity and stability. The 30 kb virulence (*vir*) region is a regulon organized in six operons that are essential for the T –DNA transfers (*virA, virB, virC* , *virD virE*, and *virG*) or for the increasing of transfers efficiency (*virF* and *virH*) (Hooykaas and Schilperoot, 1992; Zupan and Zambryski. 1995). Different chromosomal – determined genetic elements have shown their functional roles in the attachment of *A. tumefactiens* to the plant cell and bacterial colonization; the loci *chvA* and *chvB*. Involved in the synthesis and excretion of the β – 1,2 glucan (Cangelosi *et al*., 1989), several genes were identified; the *chvE* required for the sugar enhancement of *vir* genes induction and bacterial chemotaxis (Ankenbauer *et al*., 1990, cangelosi *et al*., 1990, 1991); the *cel* locus, responsible for the synthesis of cellulose fibrils (Matthysse 1983); the *pscA*

(*exoC*) locus, playing its role in the synthesis of both cyclic glucan and acid succinoglycan (Cangelosi *et al*., 1991); and the *att* locus, which involved in the cell suface proteins (Matthysse, 1987).

1. *Agrobacterium tumefaciens* T – DNA transfer process

The process of gene transfer from *Agrobacterium tumefaciens* to plant cells implies several essential steps as shown in figure 3; (1) bacterial colonization (2) induction of bacterial virulence system (3) activation of the bacterial *vir* genes and production of the transferable T strand complex (4) transport into the host plant cell and (5) integration of T – DNA into plant genome as shown in Figure 3.

1.1 Bacterial colonization

Bacterial colonization is an essential and the earliest step in tumor induction and takes place when *A. tumefaciens* is attached to the plant cell surface (Figure 4) (Matthysse, 1986). Mutagenesis studies show that non-attaching mutants loss the tumor-inducing capacity (Cangelosi *et al*., 1987; Douglas *et al*., 1982; Thomashow *et al*., 1987; Bradley *et al*., 1997). The polysaccharides of the *A. tumefaciens* cell surface are proposed to play an important role in the colonizing process. The chromosomal 20 kb *att* locus contains the genes required for successful bacterium attachment to the plant cell (Bradley, 1997).

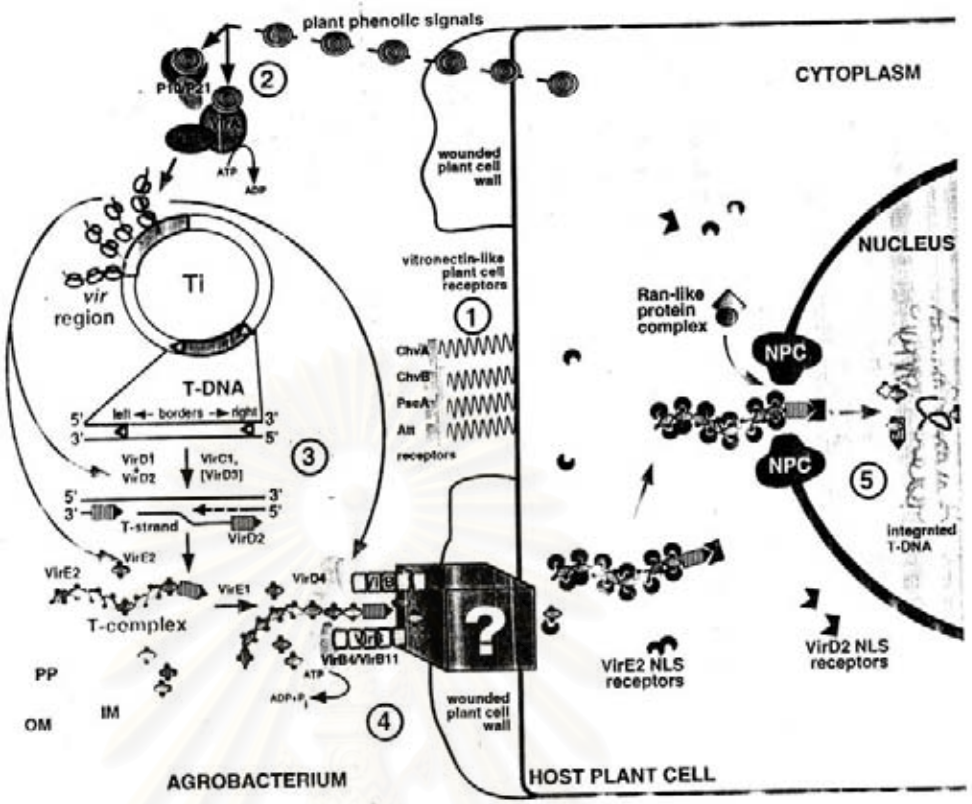


Figure 3 Agrobacterium - plant cell interaction (Sheng and Citovsky, 1996).

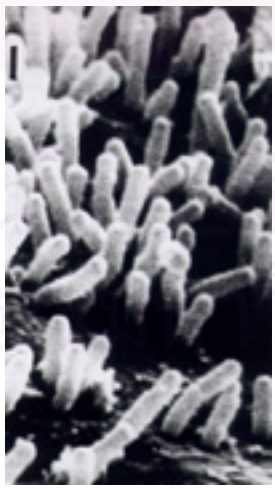


Figure 4.*A. tumefaciens* is attached to the plant cell surface (Matthysse, 1986).

1.2 Induction of bacterial virulence system

The T-DNA transfer is by products encoded by the 30-40 kb *vir* region of the Ti plasmid. This region is composed by at least six essential operons (*virA, virB, virC, virD, virE, virG*) and two non-essentials (*virF, virH*). The number of genes per operon differs, *virA, virG*, and *virF* have only one gene; *virE, virC, virH* have two genes while *virD* and *virB* have four and eleven genes, respectively. The only constitutive expressed operons are v*irA* and *virG*, coding for a two – component (*virA-virG*) system activating the transcription of the other *vir* genes. The VirA – VirG two component system has structural and functional similarities to other already described for other cellular two component system mechanisms (Nixon, 1986; Luchi, 1993).

VirA is a transmembrane dimeric sensor protein that detects signal molecules, mainly small phenolic compounds, released from wounded plants (Pan *et al*., 1993). The signals for VirA activation include acidic pH, phenolic compounds, such as acetosyringone (Winans *et al*., 1992), and certain class of monosaccharides, which acts synergistically with phenolic compounds (Ankenbauer *et al*., 1990; Cangelosi *et al*., 1990 ; Shimoda *et al*., 1990 ; Doty *et al*., 1996). Activated VirA has the capacity to transfer its phosphate to a conserved aspartate residue of the cytoplasmic DNA binding protein VirG (Jin *et al*., 1990a, 1990b; Pan *et al*., 1993). VirG function as a transcriptional factor regulating the expression of *vir* genes when it is phosphorylate by VirA (Jin *et al*., 1990a, 1990b). The C-terminal region is responsible for the DNA binding activity, while the N-terminal is the phosphorylation domain and shows homology with the VirA receiver (sensor) domain.

1.3. Activation of the bacterial *vir* genes and production of the transferable strand complex

The activation of *vir* genes produces the generation of single-stranded (ss) molecules representing the copy of the bottom T – DNA strand. Any DNA placed

between T – DNA border will be transferred to the plant cell, as single strand DNA, and integrated into the plant genome. The proteins VirD1 and VirD2 play a key role in this step by, recognizing the T – DNA border sequences and nicking (endonuclease activity) the bottom strand at each border. After endonucleotidic cleavage, VirD2 remains covalently attached to the 5' – end of ss – T – strand. This association prevents the exonucleolytic attack to the 5' – end of leading end of the T – DNA transfer complex. VirD1 interacts with the region where the ss-T – strand is originated. VirD1 is essential for the cleavage of supercoiled stranded substrate by VirD2 (Zupan and Zambryski, 1995; Christie *et al*., 1997).

1.4 Transport into the host plant cell

The transferring vehicle to the plant nucleus is an ss T – DNA – protein complex. It must be translocated to the plant nucleus passing through three membranes, the bacteria cell membrane, the plant cell membrane and nuclear membrane. According to the most accepted model, the ssT-DNA – VirD2 complex is coated by the 69 kDa VirE2 protein, a single strand DNA binding protein. This cooperative association prevents the attack of nucleases and, in addition, extended ssT – DNA strand reducing the complex diameter to approximately 2 nm, making the translocation though membrane channels easier. However, that association does not stabilizes T – DNA complex inside *Agrobacterium* (Zupan *et al*., 1996). This fact indicates that both proteins presumably play important role once the complex is in the plant cell mediating the complex uptake to the nucleus (Herrera-Estrella et al., 1990 ; Shurvinton *et al*., 1992 ; Rossi *et al*., 1993 ; Tinland *et al*., 1995 ;Zupan *et al*., 1996).

The majority of VirB proteins are assembled as a membrane –spanning protein channel involving both membranes (Shirasu and Kado, 1993; Shirasu *et al*., 1994; Stephens *et al*., 1995). Except for VirB11, they have multiple periplasmic domains (Christie, 1997). VirB1 is the only member of VirB protein found in the extracellular

milieu (Baron *et al*., 1997) although it is possible that some of the other VirB protein may be redistributed during the process of biogenesis and functioning of the transcellular conjugal channel (Christie, 1997).

It is known that VirB2 through VirB11 are essential for DNA transfer, suggesting that these proteins are fundamental component of the transfer apparatus (Berger and Chriatie, 1993, 1994).

Two accessory *vir* operons, present in the octopine Ti-plasmid, are *virF* and *virH*. The *virF* operon encodes for a 23 kDa protein that functions once the T – DNA complex is inside the plant cells via the conjugal channel or independently, as it was assumed for VirE2 export. The role of VirF seems to be related with the nuclear targeting of the ssT – DNA complex but its contribution is less important than in the case of VirF (Hooykass and Schilperoort, 1992).

The *virH* operon consists of two genes that code for VirH1 and VirH2 proteins. These Vir proteins are not essential but could enhance the transfer efficiency, detoxifying certain plant compounds that can affect the bacterial growth (Kanemoto *et al*., 1989). If that is the function of VirH proteins, they play a role in the host range specificity of bacterial strain for different plant species.

1.5. Integration of T – DNA into plant genome

Inside the plant cell, the ssT – DNA complex is targeted to the nucleus crossing the nuclear membrane. Two Vir proteins have been found to be important in this step; VirD2 and VirE2, which are the most import, and probably VirF, which has a minor contribution to this process (Hooykaas and Schilperoot, 1992). The nuclear location signals (NLS) of VirD2 and VirE2 play an important role in nuclear targeting of the delivered ss- T – DNA complex. VirD2 has one functional NLS. The ssT- DNA complex is a large (up to 20 kb) nucleoprotein complex containing only one 5'end covalently attached VirD2 protein per complex. But the complex is coated by a large

number of VirE2 molecules (approximately 600 per a 20 kb T – DNA), and each of them has two NLS. The two NLS of VirE2 have been considered important for the continues nuclear import of ss – T – DNA complex, probably by keeping both sides of nuclear pores simultaneously open. The nuclear import is probably mediated also by specific NLS binding proteins, which are present in plant cytoplasm.

The final step of T – DNA transfer is its integration into the plant genome. The mechanism involved in the T – DNA integration has not been characterized. It is considered that the integration occurs by illegitimate recombination (Lehman *et al*., 1994). VirD2 has an active role in the precise integration on T – strand in the plant chromosome (Jayaram, 1994).

2. Transformation of in some Solanaceous species by *Agrobacterium tumefaciens*

A. tumefaciens can introduce the foreign genes into many different plant species. However, the efficiency of T-DNA transfer cans very dramatically different depending on plant species, cultivars or target tissues. The latter is important, because in order to obtain a transgenic plant, it is essential that the transformed cells can be regenerated into plants. For many different plant species, methods have been described with which it is possible to obtain transgenic plants after *Agrobacterium*-mediated DNA transfer. Effective protocols use leaves, roots, hypocotyls, petioles, cotyledons, or seed as targets for transformation depending on plant species (Horsh *et al*., 1985). Large variations in efficiency may be due to differences in the tissue culture regime, conditions in which the plants are grown or the type of A*grobacterium* strain used.

Many Solanaceous plant species, such as (*Nicotiana tabaccum* L.), petunia (*Petunia hybrida* L.) and potato (*Solanum tuberosum* L.) are valuable species for biology study because they will permit the integration of the tools and concepts of genetics, plant physiology, developmental biology, host pathogen interaction, molecular biology and genetic engineering for studying and manipulating all these

processes. They are susceptible to *Agrobacterium* infection, and are therefore amenable to current plant transformation techniques. Cultured explant of Solanaceous plant species easily gives rise to shoots (McCormick *et al*., 198).

The first record on transgenic tobacco plant expressing foreign genes appeared at beginning of the last two decade (Herrera-Estrella *et al*., 1983). A well known transformation by *Agrobacterium* method is leaf disc transformation method by Horsch *et al*. (1985). It can be applied for petunia, tobacco and potato transformation (Smith, 1992; Lin *et al*, 1994; Filho, 1994). Surface-sterilized leaf disks were inoculated with an *Agrobacterium tumefaciens* strain containing a modified tumor-inducing plasmid,in which the phytohormone biosysthetic genes from transferred DNA had been deleted and replaced with a chimeric gene for kanamycin resistance, and cultured for 2 days. The leaf disks were then transferred to selective medium containing appropriate antibiotic for the selection. Shoot regeneration occurred within 2 to 4 weeks.This method for producing transformed plant combines gene transfer, plant regeneration, and effective selection for transformants into a single process. An *et al*. (1986) transfomed various plant organ pieces (leave, cotyledon, hypocotyl and stem) of four *Nicotiana* species, seven tomato cultivars, two potato cultivars and *Arabidopsis thailiana* by *Agrobacterium tumefaciens* cells,carrying the binary Ti vector , pGA472, and helper Ti plasmid, p TiBo542 or pAL4404. Transformants were regenerated and selected on MS agar medium containing 200 mg/L of kanamycin and 500 mg/l of carbenicillin with the following supplement: 0.5 mg/L of BA for tobacco shoot induction; 0.5 mg/L of BA and 15 mg/L of glutamine for potato shoot induction ; 2 mg/L of 2,4-D for tomato callus and 0.5 mg/L of 2,4-D and 0.05 mg/L of kinetin for *A. thaliana.*

Several years ago, many scientists have selected Solanaceous plant species for study foreign genes or tissue specific promoters expression by Agrobacterium-mediated transformation method, such as Kosaki *et al* (1992) transformed promoter of

RGS-38 gene, which encodes plastidic glutamine systhetase of *Oryza sativa* L., fused to a β-glucuronidase (GUS) reporter gene, into tobacco plants. The promoter directed GUS expression, both leaves and roots, and the expression of GUS was reglulated by light. Perl *et al* (1993) transformed tomato Cu,Zn superoxide dismutases into potato tuber discs. Several transgenic potato lines showed elevated tolerance to the superoxide-generting herbicide paraquat (methyl viologen). Filho *et al* (1994) transformed pGV1040 plasmid, which contained marker gene for resistance to kanamycin and phosphinothricin plus the gene for enzyme β-glucuronidase, into leaf of three potato cultivars. The transgenic potatoes could be resistant to phosphinothricin both *in vitro* and greenhouse culture. Mol *et al* (1995) transformed a maize *dihydroflavonol 4-reductase* gene (*dfr*) into *Petunia hybrida* L. In many of transformants, the flower color was pale brick red, but this was unstable, because of epigenetic silencing of transgene. Terakawa *et al* (1997) transformed a fungal chitinase (*chi1*) from *Rhizopus oligosporus* into leaf disc of tobacco plants. Chitinase activity in the extracts of young leaves from transgenic tobacco was three- to- four fold higher than control plant. A fungal infection assay on leaves infected with dicomycete pathogens was remarkably suppressed as compared with the control leaves.

III. GUS assay

A *gus* reporter gene system was developed to meet several criteria required for the use in plants, such as easy quantification, high sensitivity, sufficient specificity of enzymatic reaction to minimize interferance with normal cellular metabolism, possibility of histochemical localization of reaction, and activity of the enzyme in translational fusion. Substrates for GUS are available for spectrophotometric, fluorometric, and histochemical assay (Schrott, 1995).

3.1 The *gus* operon in *E. coli.*

The gene encoding β-glucuronidase , *gusA* (formerly *uidA*) , which is now widely used as a reporter gene in plants and other organisms, was originally isolated from *Escherichia coli* (Jefferson *et al*., 1986). Maps at minute 36 on the *E. coli* chromosme, between the loci *add* (adeninedeaminase) and *manA* (mannose-6-phosphate isomerase) (Novel and Novel, 1973). It has become clear that other genes involved in glucuronide metabolism and in regulation of β-glucuronidase activity map to the same region of the *E. coli* chromosome, forming the *gus* operon. The region has been studied extensively at a genetic and molecular-genetic level, and the current working model of the structure and functioning of the *gus* operon is summarized in Figure 5 (Wilson *et al*, 1992).

This regulation is due to the action of the product of the *gusR* (formerly *uidR*) gene, which encodes a repressor that is specific for the *gus* operon. Inactivation or deletion of *gusR* leads to constitutive β-glucuronidase activity. The *gusR* gene maps to the same region of the chromosome as *gusA*, lying upstream of *gusA* and being separately transcribed. The direction of transcription is the same as *gusA* (Wilson *et al*., 1992)

3.2 Substrates for GUS activity detection

Various β-glucuronic acid substrates are available for detection of GUS expression *in vivo* and *in vitro*. All of these substrates contain the sugar D-glucopyranosiduronic acid attached by glycosidic linkage to a hydroxyl group (usually a phenolic hydroxyl) of a chromogenic, fluorogenic, or other detectible molecules (Naleway, 1992).

3.2.1 Quantitation of GUS activity by fluorometry

The most widely used fluorogenic substrate for detection of β-glucuronidase activity *in vitro* is 4-methylumbelliferyl β-D-glucuronide (4-MUG). Upon hydrolysis by GUS, however, the fluorochrome 4-methylumbelliferone (7-hydroxy-4-methyl coumarin) is produced along with sugar glucuronic acid. Using excitation at 363 nm and measuring emission at 447 nm, background fluorescence from the substrate is negligible. In general, measurements of GUS activity using this fluorescent substrate are two to three orders of magnitude more sensitive than X-Gluc (5-bromo-4-chloro-3-indodyl β-D-glucuronide: a substrate for histochemical detection) or pNPG (ρara-nitrophenyl β-D-glucuronide : a substrate for spectrophotometric detection).

The fluorometric assay on the hydrolysis of the MUG substrate by GUS, with the reaction

4-methylumbelliferyl β-D-glucoronide (MUG) ⟶ glucolonic acid

+

7-hydroxy-4-methylcoumarin (MU)

The 7-hydroxy-4-methylcoumarin (4-methylumbelliferone; MU) is maximally fluorescent when the hydroxy is ionized, and as a consequence the assays are stopped with sodium carbonate (Gallagher, 1992).

3.2.2 Histochimical localization of β-glucuronidase

5-Bromo-4-chloro-3-indolyl B-D-glucuronide (X-Gluc) is generally ecommended for histochemical localization of GUS activity precipitation of the product in aqueous solutions provides a convenient way of localizing enzyme activity *in vivo*. This colorless substrate produces a blue indigo dye precipitate at the site of enzymatic cleavage. Color formation requires three separate reactions. After enzymatic

turnover, the released indoxyl derivative dimerizes and is then oxidized to the final indigo dye (Figure 6).

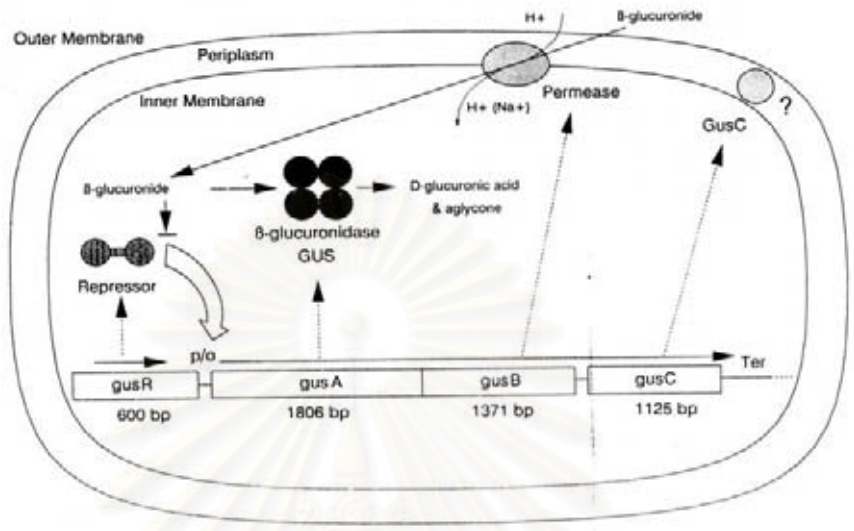


Figure 5. The *gus* operon in *E.coli* (Wilson *et al*, 1992).

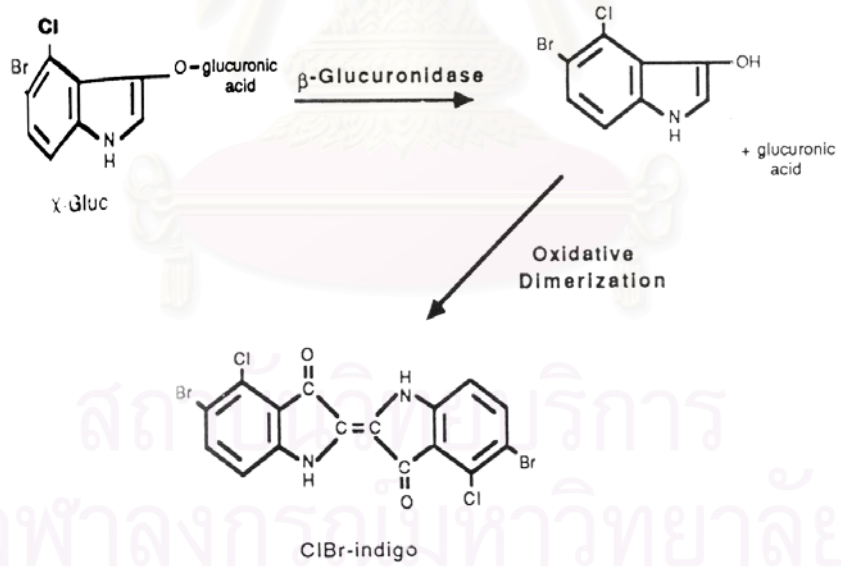


Figure 6. Clevage of 5-Bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) produces the final insoluble blue precipitate dichloro-dibromoindigo (ClBr-indigo) (Stomp, 1992).

CHAPTER III

MATERIALS AND METHODS

I. MATERIALS

1. Plant materials

1.1 Tabacco (*Nicotiana tabacum* L. cultivar Virginia Coker)

Nicotiana tabacum L. cultivar Virginia Coker 347 is a tobacco generally planted in Thailand. After surface sterilization of seed stock and seed germination, tobacco plants have been aseptically maintained in MS complete medium in the plant tissue culture room at the Department of Botany, Faculty of Science. Chulalongkorn University.

1.2 Petunia (*Petunia hybrida* L.)

The commercial petunia hydrid, seeds were purchased from Yate company. The seeds, then were surface sterilized and aseptically grown in MS complete medium in the plant tissue culture room at the Department of Botany, Faculty of Science , Chulalongkron University.

1.3 Potato (*Solanum tuberosum* L. cultivar Spunta)

Solanum tuberosum L. cultivar *Spunta* is the common potato cultivar for Thai agriculture. The original aseptic potato plantlets were regenerated from tubers. Then the potato plants have been maintained in the plant tissue culture room at the Department of Botany, Faculty of Science, Chulalongkorn University.

2. Bacterial strain

2.1 *Agrobacterium tumefaciens* strain LBA4404 – ProA1.1

Agrobacterium tumefaciens strain LBA4404, the appropriate strain for Agrobacterium transformation with the binary system, generously provided by Dr. Milton Gordon, Department of Biochemistry, University of Washington. *A. tumefaciens* strain LBA4404 – ProA1.1 contains ProA1.1 plasmid which is derived from PBI101.2 (Clontech) by *Arabidopsis TGG1* promoter in frame insertion at the upstream region of β-glucuronidase (*gus*) gene to regulate the *gus* reporter gene. The map of the T-DNA of this strain is show in Figure 7.

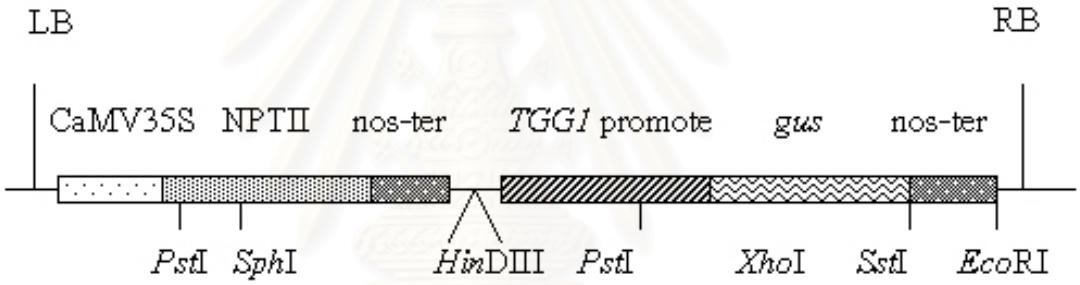


Figure 7 Chimeric construct of *Arabidopsis TGG1* promoter- *gus* gene in *A. tumefaciens* strain LBA4404 – ProA1.1

2.2 *Escherichia coli* strain DH5∝ - PBSProA1.1

This bacterial strain contains the PBS-ProA1.1 plasmid. ProA1.1, an 8 kb-long plasmid,was cloned into pBluescript KS + as an EcoRI fragment , given PBSProA1.1 plasmid. The map of PBS ProA1.1 is shown in Figure 8.

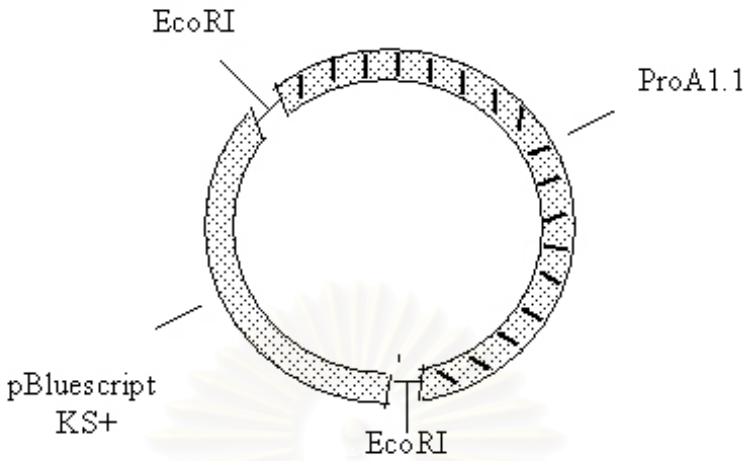


Figure 8 The map of PBS ProA1.1

3. Lab Equipment

- Spectophotometer (Spectronic, Genesys 5)
- Centrifuge (Universal16,Hettich, Germany)
- Autoclave
- Fluorometer (DyNA Quant 200, Hoefer)
- DNA
- RNA gel electrophoresis apparatus
- shaking water bath
- hot air oven
- hot plate and magnetic stirrer
- laminar air flow
- pH meter
- auto pipette
- vacuum pump
- shaker
- microscope (Nikon, LABOPHOT-2)

4. Glasswares, plasticwares and tools

- Glass bottles
- Forceps
- Petri-dishes (9 cm diameter)
- Erlenmeyer flask
- autopipette tip
- Eppendorf tubes
- Centrifuge tubes
- 50 ml test tubes
- Beakers
- Cylinders
- Parafilm
- Loop
- Pots
- Tags
- Icebox
- Mortars and pestles
- Spatula
- Glass trays
- Plastic bags
- vials
- Suction flask

5. Plant tissue culture room

The plant tissue culture room temperature is controlled at 25 ^{o}C under 16 hour light and 8 hour dark cycle illumination with 36 watt fluorescent light. The light intensity, measured at the plant surface is 35 μmol $m^{-2}s^{-1}$.

6. Chemicals

6.1 Chemicals for seed sterilization

- Clorox (20 % sodium hypochlorite)
- Tween 20
- Sterilized distilled water

6.2 Chemical for plant tissue cultre and *Agrobacterium* ─ mediated transformation

- MS complete stock solution (see in appendix I)
- sucrose
- myo-inositol
- hormones
    1. BAP (6 ─ benzylaminopurine)
    2. IAA (indole acetic acid)
    3. Kinetin
    4. NAA (napthaleneacetic acid)
    5. Zeatin
- Antibiotics
    1. Kanamycin
    2. Carbenicillin
- Mg/L medium (see in appendix I)

6.3 Chemicals for DNA analysis

- Dellaporta's extraction buffer (see appendix I)
- Isopropanol
- Liquid nitrogen (from TIG, Thailand)
- 5 M Potassium acetate (CH_3COOK)
- 70 % Ethyl alcohol

- TE buffer (see Appendix I)
- 3 M Sodium acetate (CH_3COONa) pH 5.1
- Sterilized distilled water
- Qiagen Midi Kit chemical (see Appendix I)
- Agarose gel
- 5 x TBE (see Appendix I)
- DNA Loading buffer (see Appendix I)
- Restriction enzymes (Biorabs)
    1. *Eco*RI
    2. *Hin*dIII
    3. *Xho*I
- 20x SSC (see Appendix I)
- Denaturation solution (see appendix I)
- Neutralization solution (see appendix I)
- 0.2 N Hydrocholric acid
- ECL direct nucleic acid labeling and detection system, RPN 3001, Amersham International, Amersham, UK.
- X-ray film developer (Kodak)
- X-ray film fixer (Kodak)

6.4 Chemicals for GUS assay

- X- Gluc reagent mix (see Appendix I)
- 2 mM MUG (see Appendix I)
- 1 mM MU standard (see Appendix I)
- GUS extraction buffer (see Appendix I)
- 0.2 N Na_2CO_3 (Carbonate stop buffer) (see Appendix I)
- total protein assay (Biorad Co.)

II. METHODS

1. Determination of *Agrobacterium tumefaciens*. LBA 4404 - Pro A1.1 growth on Mg/L medium.

The best plant transformation process can be obtained when the *Agrobacterium* at the late log phase stage is used for the transformation. Therefore, *Agrobacterium* growth curve was determined to indicate the appropriate time period for log phase harvested *Agrobacterium* for the transformation process.

A. tumefaciens LBA4404 ─ ProA1.1 was grown on 50 mg/L kanamycin Mg/L agar plate for 3-4 days at 28°C. A single colony with diameter of 0.1 cm was picked and resuspended in 25 ml Mg/L broth in a 125 ml flask. The overnight grown liquid culture at 28°C was used as a starter. One ml inoculum of the starter was inoculated in 25 ml Mg/L freshly prepared broth and incubated at the same condition. The cell concentration was determined at time 0, 6, 12 , 18 , and 24 hours after inoculation. Two replicates were done at each time period.

2. *Agrobacterium* transformation in Solanaceous plant species.

2.1 Tobacco

Tobacco transformation was done according to Lin *et al*. (1994) with some modifications for *Agrobacterium* preparation.

2.1.1 *Agrobacterium* preparation

Agrobacterium cells was streaked on Mg/L agar plate, supplemented and incubated atwith 50 Mg/L kanamycin at 28 °C for 3-4 days. A single colony resuspened in 25 ml Mg/L broth and incubated and incubated at 28 °C overnight on a shaker. One ml of the overnight grown culture was reinoculated in 25 ml of fresh Mg/L broth and incubated in the same condition for 12-18 hours to obtain late log

phase cell culture. *Agrobacterium* cells for the transformation process were centrifuged at 3500 x g for 7 min, and the cell pellet was resuspended in MS medium containing 0.5 mg/L MES at the *Agrobacterium* concentration of $10^9 - 10^{10}$ cells / ml after OD_{620} was measured and concentration was estimated.

2.1.2 Co-cultivation

Young leaves from one-month–old plants were cut into 1 x 1 cm^2 pieces. The tobacco leaf discs were incubated with the prepared *Agrobacterium* cells (from 2.1.1) for 10 min. Then, the leaf discs were transferred to MS complete medium and co-cultivated for 2 days in tissue culture room.

2.1.3 Transgenic plant regeneration

After co-cultivation, the leaf discs were transferred to shoot induction medium, MS medium supplemented with 0.5 mg/L BA, 100 mg/L kanamycin, and 500 mg/L carbenicillin. After 3-4 weeks, small green shoots were developed to complete shoots. Then, the plantlets were transferred to MS complete medium for root nduction.

2.1.4 Transgenic plant hardening for natural grown condition.

After 3-4 weeks in the MS medium, the transgenic shoot regenerated roots. One – month old transgenic plants then were transferred out of the plant tissue culture room for 7 days of hardening period. After hardening, the media was removed by tap water and the plants were grown in sterilized soil under the natural condition.

2.2 Petunia

Petunia transformation via *A. tumefaciens* was done according to Smith (1992). The procedure is as followed.

2.2.1 Preculture of petunia leaf tissue.

Young leaves from one-month–old plants were cut into 1 x 1 cm^2 pieces and percultured on the semisolid MS medium, containing no B5 vitamins and supplemented with 1.0 mg/L BA and 0.1 mg/L NAA for 2 days under plant tissue culture condition as previously indicated.

2.2.2 *Agrobacterium* preparation

Agrobacterium preparation for petunia transformation was done in the same process as the cell preparation for tobacco transformation (2.1.1).

2.2.3 Co-cultivation

The precultured petunia leaf discs were incubated with *Agrobacterium* suspension in MS liquid medium, supplemented with 0.5 mg/L MES for 10 minutes. Then they were transferred to the new bottles of preculture medium and co-cultivated in the dark at 25 ^{o}C for 2 days.

2.2.4 Transgenic plant regeneration

After co-cultvation, the leaf discs were transferred to MS medium supplemented with 1.0 mg/L BA, 0.1 mg/L NAA, 50mg/L kanamycin and 200 mg/L carbenicillin. After 3-4 weeks, small green shoots were developed to complete shoots. Then the shoots were transferred to MS medium for root induction. The roots developed in 3-4 weeks.

2.2.5 Transgenic petunia hardening for natural grown condition.

Transgenic petunia hardening for natural grown condition was done in a similar manner as what done for transgenic tobacco.

2.3 Potato

Due to the difficulty of potato transformation (An *et a.l*, 1986), two procedures of potato transformation were compared for the transformation efficiency.

2.3.1 *Agrobacterium* preparation

A. tumefaciens , containing the appropriate plasmid, was prepared similar to the procedure for *Agrobacterium* preparation for petunia and tobacco transformation.

2.3.2 Co-cultivation

Potato tissue used for transformation was obtained from the potato aseptic culture after subculturing for one month. Potato stem were cut into 128 pieces with the length of 0.5 ─ 1.0 cm. Then, stem pieces were incubated in the *Agrobacterium* cell suspension, prepared as described in 2.2.1, for 10 minutes. A half of explants (64 stem pieces) were transferred to the MS medium, supplemented with 1.0 mg/L Zeatin, 0.01 mg/L NAA, while the others were transferred to the MS medium, supplemented with 1.0 mg/L Zeatin for 2 day co-cultivation in the dark.

2.3.3 Transgenic plant regeneration

After co-cultivation, the explants were moved to the selective media. The media containing the same amount of plant hormones as those for co-cultivation process were used while 50 mg/L kanamycin and 200 mg/L carbenicillin were also added to each medium for transgenic selection and *Agrobacterium* elimination, respectively. After potato shoots were regenerated, then they were transferred to root

induction medium, MS medium supplemented with 0.1 mg/L IAA, 0.1 mg/L kinetin and 50 mg/L kanamycin.

3. Determination of the inserted fragment numbers and independence of the tobacco transgenic lines.

3.1 Inserted fragment number determination by genomic Southern blot analysis.

Genomic DNA was isolated from leaves of the first generation transgenic tobacco plants using the method of Dellaporta *et al* . (1983) (Appendix I). The DNA was double digested with *Hind*III and *Eco*RI. Then, the digested DNA fragments were separated on a 0.8 % agarose gel and transferred to Hybond^{TM} – N^{+} (Nylon) filter by using the method of Sambrook *et al* (1989).

The whole ProA1.1 fragment was used as a probe. PBS ProA1.1 plasmid was purified by using Quigen column according to the manufacture procedure. Then it was digested with *Eco*RI and the 5 kb *Eco*RI fragment was isolated and used as probe. ECL^{TM} direct nucleic acid labeling system was used for probe labeling, according to the manufacturer protocol.

The filters were hybridized for 16 hours at 42 ^{o}C in gold hybridized buffer, 5% blocking reagent and 0.5 M NaCl. Each of filters was washed twice in primary wash buffer at 42 ^{o}C for 20 minute and twice in secondary wash buffer (details of the wash buffer) at room temperature for 5 minute each times. The filter, then, were exposed on Hyperfilm-ECL for 30 minute. After, exprosure, the Hyperfilm was developed and fixed with Kodak developer and fixer respectively, to obtain the signal.

3.2 Inserted fragment determination by kanamycin trait in the second generation of the transgenic tobacco lines (T_2 tobacco).

The first generation of the transgenic tobacco plants (T_1) were grown in soil. When flowering, the inflorescences were covered with the paper bags to let the transgenic plants self-fertilized. Then, seeds (T_2 seeds) were collected separately for each line. T_2 seeds from ten putative independent lines of transgenic tobacco were used for kanamycin resistance trait segregation test. At least 100 seeds from each lines were surface sterilized with 70 % ethanol for 1 minute and followed with 20 % commercial bleach (chlorox), containing a few drops of TWEEN 20 for 10 minutes. Then, they were rinsed with sterilized water for 5 times. The sterilized seeds were planted on MS medium, containing 50 mg/L kanamycin for one month. The numbers of resistant and sensitive plants were recorded for each line. Chi-square test was used to determine the number of positive kanamycin resistant gene insertion.

4. Quantitation of *gus* gene expression regulated with *Arabidopsis TGG1* promoter in transgenic tobacco.

Fluorometric assay for GUS enzyme was performed, according to Gallagher (1992). Briefly, plant tissues were homogenized in GUS extraction buffer (Gallagher, 1992) and centrifuged at 3500 xg for 3 minutes. The supernatant was used for GUS activity assay by measuring the amount of fluorometric products, released from MUG (4-methylumbelliferone glucuronide). Protein concentration in the extract was determined by using Bio-Rad D^C Protein assay in comparison with the BSA standard proteins. The details of GUS fluorometric protocol is shown in Appendix II. Each protein extract was assayed twice for GUS activity, and the average data were used for the results. GUS activity was indicated in the unit of pmole MU/min/mg protein.

GUS activity in different organs of T_1 and T_2 transgenic lines was measured with fluorometric assay (Gallagher, 1992). The transgenic line no. T7 was used as the representative for *gus* gene expression in the transgenic tobacco lines.

The non-transformed tobacco leaf tissue was used as the negative control for every assay.

4.1 *Gus* gene expression regulated by *Arabidopsis TGG1* promoter in T_1 transgenic tobacco.

4.1.1 Expression in leaves

After the T_1 transgenic tobacco line T7 was planted in soil for one month, *gus* gene expression in leaf tissues was determined. Tissues from leaf 1 to 12 (top to bottom) were taken and GUS activity was measured fluorometrically as previously described.

4.1.2 Expression in reproductive organs and immature fruits

The T_1 transgenic tobacco line T7 was soil grown. One and a half month after the transfer from the tissue culture condition, the transgenic plants started producing reproductive organs. Floral tissues at 4 different ages, 4, 7, 10 and 15 day-old after floral bud formation, were taken for GUS activity assay. Floral tissues, used in the experiment, were from sepal, petal, corolla tube, corolla lobe, anther, filament, stigma, style, and ovary (or pericarp of young fruit), including the pedicel.

4.2 *Gus* gene expression regulated by *Arabidopsis TGG1* promoter in T_2 transgenic tobacco.

T_2 seeds of transgenic tobacco line T7 were aseptically planted in the MS medium, containing 50 mg/L kanamycin for the kanamycin resistant trait selection. The kanamycin resistant seedlings were used for the GUS activity assay.

Each experiment was performed with Completely Randomized Design (CRD) with four replications.

4.2.1 Expression in cotyledons

Cotyledons of the T_2-transgenic seedlings at the age of 3, 6, 9, 12 and 15 days after germination were harvested and GUS activity was assayed as previously described.

4.2.2 Expression in leaf tissues

The *gus* gene expression was determined in leaf tissues at different ages, taken from aseptically grown plants and naturally soil grown plants.

For aseptically grown plants, the 2-week old kanamycin resistant seedlings were maintained in aseptic condition for another 6 weeks. Tissues from leaf 1 to 14 were harvested and GUS activity was assayed as previously described.

For the naturally soil grown plants, tissues from leaf 1 to 12 of the 8 week-old plants, were harvested for GUS activity assay with the same method.

4.2.3 Expression in reproductive organs

Floral tissues, sepal, petal, corolla tube, corolla lobe, anther, filament, stigma, style, and ovary, including pedicel, at 4 different developmental stages, 4, 7, 10 and 15 day-old-after floral bud formation were harvested from 10 week-old, soil grown tobacco line T7, and GUS activity was determined fluorometrically. No

replications could be done in this study, because there was not enough material of the same age at during the experiment. Different stages of flowers were used for analysis.

5. Histochemical localization of GUS enzyme under *Arabidopsis TGG1* promoter regulation in transgenic tobacco.

Ten of the first generation transgenic tobacco line, T1-T10, were used for GUS localization. The transgenic plants were soil grown for 8 weeks and the tissues of the third leaf from the top of each plant and stem were taken for GUS histochemical assay. Floral organs were taken from the transgenic lines, naturally soil grown for 10 weeks.

GUS histochemical assay was performed according to Stomp (1992). The harvested tissues were prepared by sectioning or epidermal peeling and then submerged in the GUS staining solution (Stomp, 1992). Vaccuum infiltration was done to let the substrate penetrate into the tissues equally. Then, the tissues were incubated at 37 ^{o}C for 5 to 24 hours. Green plant tissues were incubated in 70 % and 95 % ethanol respectively to remove the chlorophyll, if needed. Before investigation with light microscope, the tissues were rehydrated. Photographs were taken with Nikon camera and Fuji superia 200 films.

6. Quantitation of *gus* gene expression regulated by *Arabidopsis TGG1* promoter in transgenic petunia.

After the transfer of transgenic petunia plants from the aseptic growing condition to soil grown condition for 4 weeks, the transgenic petunia started flowering. Tissues from the third leaf from the top, and floral organs from 5 day-old after floral bud formation, calyx, corolla, stamen, pistil and pedicel, were harvested and fluorometrically assayed for GUS activity, as described previously. The non-transformed petunia leaf tissue was used as the negative control.

7. Histochemical localization of GUS enzyme under *Arabidopsis TGG1* promoter regulation in transgenic petunia.

The first generation transgenic petunia line -P1 was used for GUS histochemical localization experiment. Tissues taken from the third leaf, and floral bud, aged 7 day-old after floral bud formation, were used for GUS localization study. Plant tissues were epidermal peel, whole organ and sections which were then prepared by the GUS staining process (Stomp, 1992) as previously described.

8. Quantitation of *gus* gene expression regulated by *Arabidopsis TGG1* promoter in transgenic potato.

Nine of aseptically grown transgenic potato line, Pzn 1, Pzn 2, Pzn 3, Pz 1, Pz 2, Pz 3, Pz 4, Pz 5, and Pz 6, were used for GUS fluorometric assay. The transgenic plants were maintained in MS medium, supplemented with 0.1 mg/L IAA and 0.1 mg/L kinetin for one month, before leaf and stem tissues were harvested. GUS fluorometric assay was done as previously described. The non-transformed potato tissue, maintained in the same condition was used as a negative control.

9. Histochemical localization of GUS enzyme under *Arabidopsis TGG1* promoter regulation in transgenic potato.

Tissues from the aseptically grown transgenic potato lines were submerged into GUS staining solution (Stomp, 1992) and vaccuum infiltrated. The tissues were incubated at 37 ^{o}C for 3 days. Then, they were investigated for GUS localization under the microscope.

CHAPTER IV

RESULTS

1. Determination of *Agrobacterium tumefaciens* LBA4404-ProA1.1 growth on Mg/L medium.

After 1 ml inoculum of overnight grown *A. tumefaciens* cells culture in Mg/L medium at 28 ^{O}C , cell concentration was determined at time 0, 6, 12, 18 and 24 hours after inoculation. The growth curve of *A. tumefaciens* during 24 hours was shown in figure 10.

Base on the growth curve pattern, the log phase was ended in 12 hours after inoculation. However, the beginning log phase was not clearly seen, because time interval for cell concentration determination was too long.

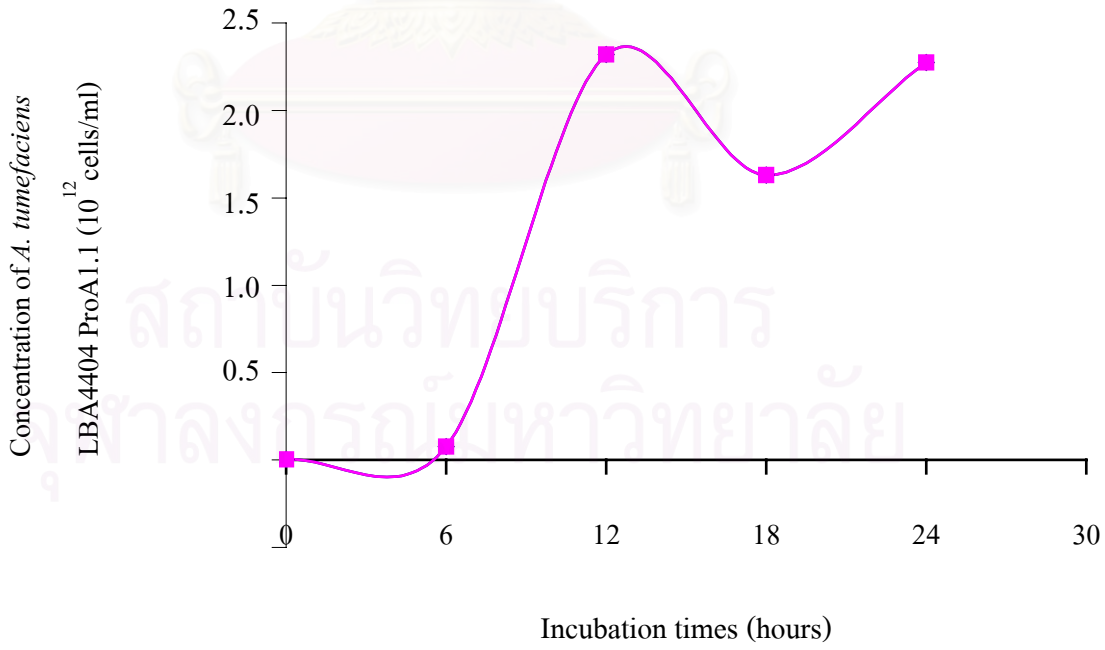


Figure 9 The growth curve of *Agrobacterium tumefaciens* LBA4404 ProA1.1 in Mg/L medium.

***2. Agrobacterium* transformation in Solanaceous plant species.**

2.1 Tobacco

Fifty leaf segments were used for transformation (Lin et al, 1994). After four weeks in selective medium, 36 pieces of leaf tissues were regenerated into transgenic shoots (Fig 10 c). The non-transformed leaf tissues were also cultured in the selective medium as the negative controls (Fig 10 a) for selective medium property, which could not regenerated and the transformed leaf tissues were also cultured in the selective medium but not added both of antibiotics as the positive controls (Fig 10 b) for transformation property, which could be regenerated. The difference of the transformed and non-transformed tissues in the selective medium was shown in figure 10.

Figure 10 Four week-old tobacco explants on semisolid MS medium supplemented with 0.5 mg/L BAP , 100 mg/L kanamycin and 500 mg/L carbenicillin.

a) non transformed leaf tissue in shoot induction medium with antibiotics
b) transformed leaf tissue in shoot induction medium without antibiotics
c) transformed leaf tissue in shoot induction medium with antibiotics

Each transgenic shoot was subsequently transferred to semisolid MS complete medium for root induction (Figure 12) for two weeks. After one week haedening, then transgenic plants were transferred to pots containing sterilized soil, and grown under natural photoperiod in greenhouse. Finally, 10 complete transgenic tobacco plants, called ProA T1-T10, were obtained for further study.

Figure 11 *In vitro* cultured transgenic tobacco plants.

Figure 12 Transgenic tobacco plants in the greenhouse.

2.2 Petunia

Eighty pieces of leaf segments were used for transformation (Smith, 1992). About four weeks, 70 pieces of leaf segments regenerated into transgenic shoots. However, the non-transformed leaf tissues, turned yellow and died after four weeks in the selective medium. The difference of the transformed and non-transformed tissues was shown in figure 13. The transgenic shoots were subsequently transferred to semisolid MS complete medium, containing 50 mg/L kanamycin, for root induction (Figure 14)

Then transgenic plants were transferred to pots containing sterilized soil and grown under natural photoperiod in greenhouse (Figure 15). We received 3 complete transgenic petunia, which were called ProA P1-P3 for further analysis.

Figure 13 Four week-old petunia explants on semisolid MS medium supplemented with 1.0 mg/L BAP , 0.1 mg/L NAA, 50 mg/L kanamycin and 500 mg/L carbenicillin

a) non-transformed leaf tissue in shoot induction medium with antibiotics
b) transformed leaf tissue in shoot induction medium without antibiotics
c) transformed leaf tissue in shoot induction medium with antibiotics

Figure 14 *In vitro* cultured transgenic petunia plants.

Figure 15 Transgenic petunia in the greenhouse.

2.3 Potato

To determine the appropriate medium for potato transformation, two kinds of medium ; formula I : semisolid MS medium, supplement with 1.0 mg/L zeatin and 0.1 mg/L NAA (ZN) and formula II : semisolid MS medium, supplement with 1.0 mg/L zeatin (Z), were used for co-cultivation process. After the co-cultivation, potato segments were transferred to the selective medium of the same formula, which 50 mg/L kanamycin and 200 mg/L carbenicillin were added order to select the transgenic shoots and eliminate *Agrobacterium* overgrow, respectively.

Sixty four potato stems segments were test for each medium formula. The non-transformed potato segments were also used as the controls of the influence of kanamycin concentration used for the transgenic plant selection. After seven weeks in the selective regenerating medium, some explants regenerated single complete shoots from both or either end of the stem segments (Figure 16). The new shoots, then, were transferred to 0.1 mg/L IAA , 0.1 mg/L kinetin and 50 mg/L kanamycin for root induction (Figure 17).

At the end of the transformation process, three complete putative transgenic potato plants were obtained from the selective medium formula I, and six putative transgenic potato plants were obtained from the selective medium formula II.

The putative transgenic potato lines from formula I were named Pzn1, Pzn2 and Pzn3, while the other six putative transgenic potato lines were called Pz1 ─ Pz6, respectively.

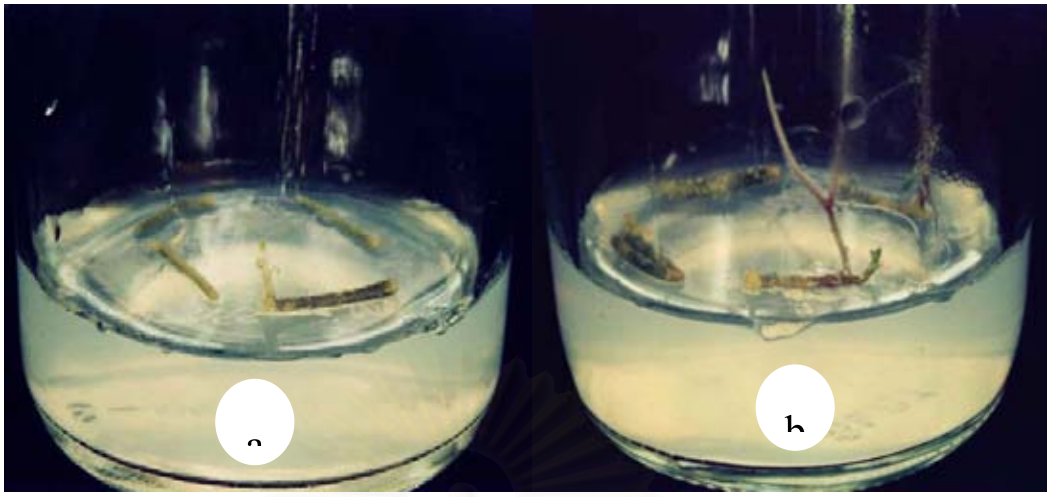


Figure 16 Potato explants in the selective medium for seven weeks.

a. MS medium containing 1.0 mg/L zeatin , 0.1 mg/L NAA, 50 mg/L kanamycin and 200 mg/L carbenicillin.

b. MS medium containing 1.0 mg/L zeatin ,50 mg/L kanamycin and 200 mg/L carbenicillin.

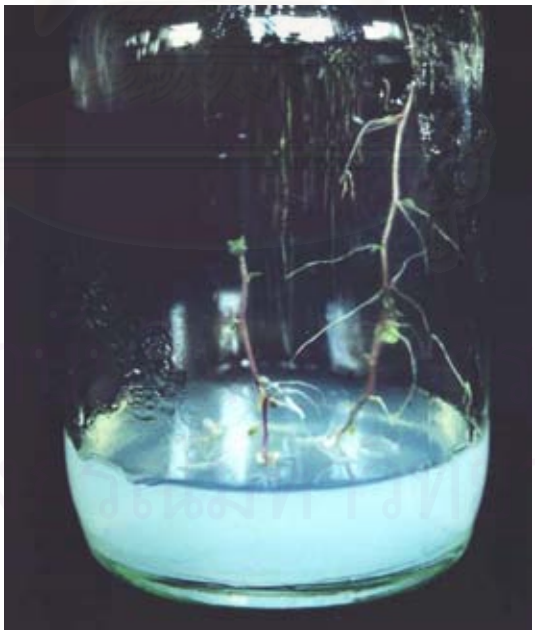


Figure 17 Independent lines of transgenic potatoes in semisolid MS complete medium supplemented with 0.1 mg/L IAA , 0.1 mg/L kinetin and 50 mg/L kanamycin for root induction.

3. Determination of the inserted fragment numbers and independence of the tobacco transgenic lines.

3.1 Inserted fragment number determination by genomic Southern blot analysis.

The genomic DNA of transgenic tobacco lines, T1 - T10, which was digested with *Hind*III and *Eco*RI were separated on a 0.8 % agarose gel. The whole Pro A1.1 plasmid were used as a probe for Southern blot analysis.

Besed on the southern blot, most of the transgenic tobacco line DNA pattern showed two positive bands ; line T1, T2, T3, T6, T7, T8 and T9. T4 and T10 showed three positive bands and T5 showed only a single positive band. However, the consistent band among these line was the approximate 3 kb fragment (Figure18).

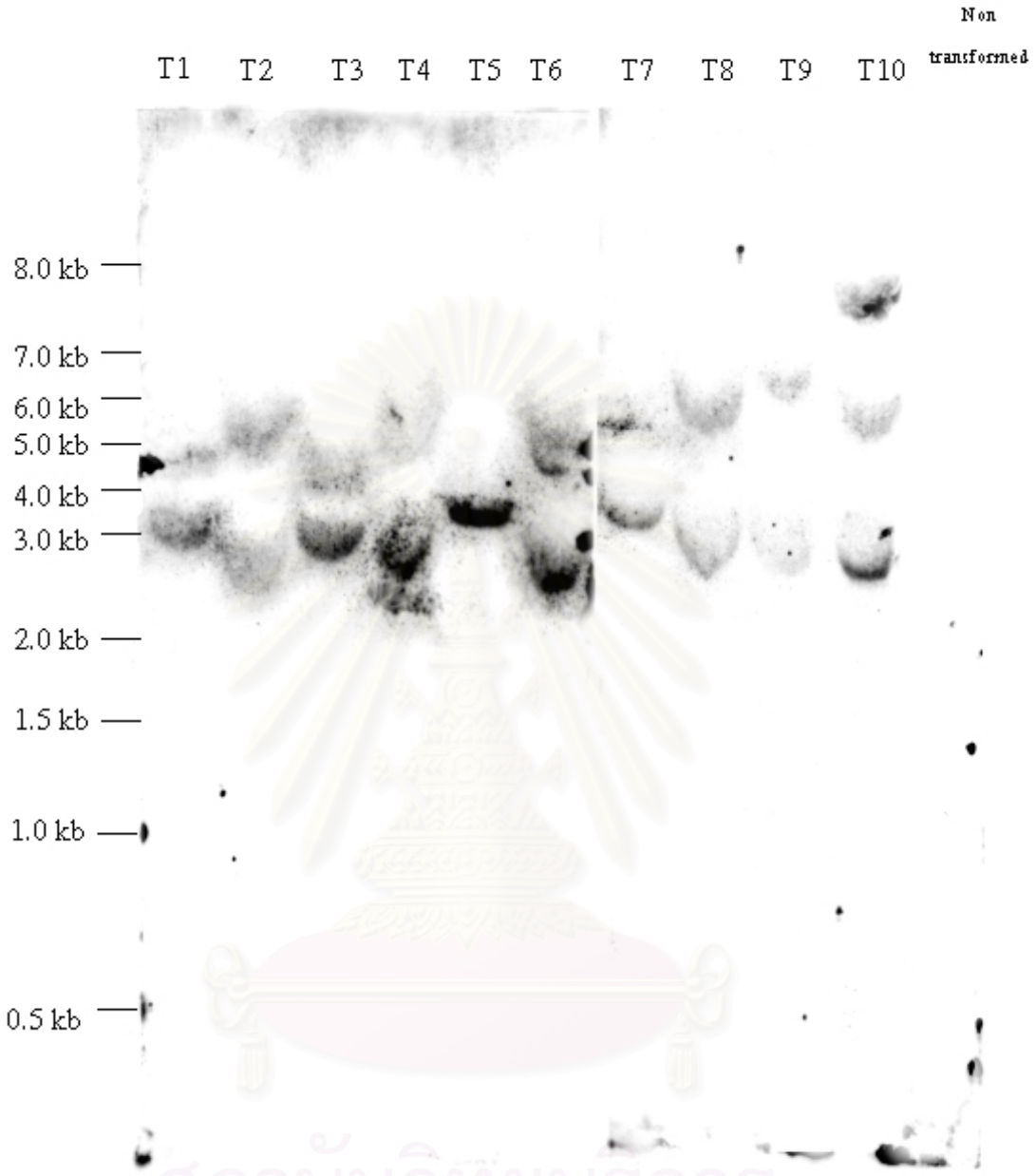


Figure 18 The genomic Southern blot of 10 transgenic tobacco lines, probed with linearization ProA1.1 plasmid.

3.2 Inserted fragment determination by kanamycin resistant trait in the second generation of the transgenic tobacco lines (T_2 tobacco).

The T_2 transgenic seeds of each lines were aseptically planted on the semisolid MS medium, containing 50 mg/L kanamycin for one month. The kanamycin resistant and kanamycin sensitive seedlings showed different characters. The kanamycin resistant seedlings performed normal growth and the first pair of true leaves was developed. The kanamycin sensitive ones had only the cotyledon which the root growth was inhibited. The seedlings of transgenic tobacco grown on MS medium containing 50 mg/L kanamycin were shown in Figure 19.

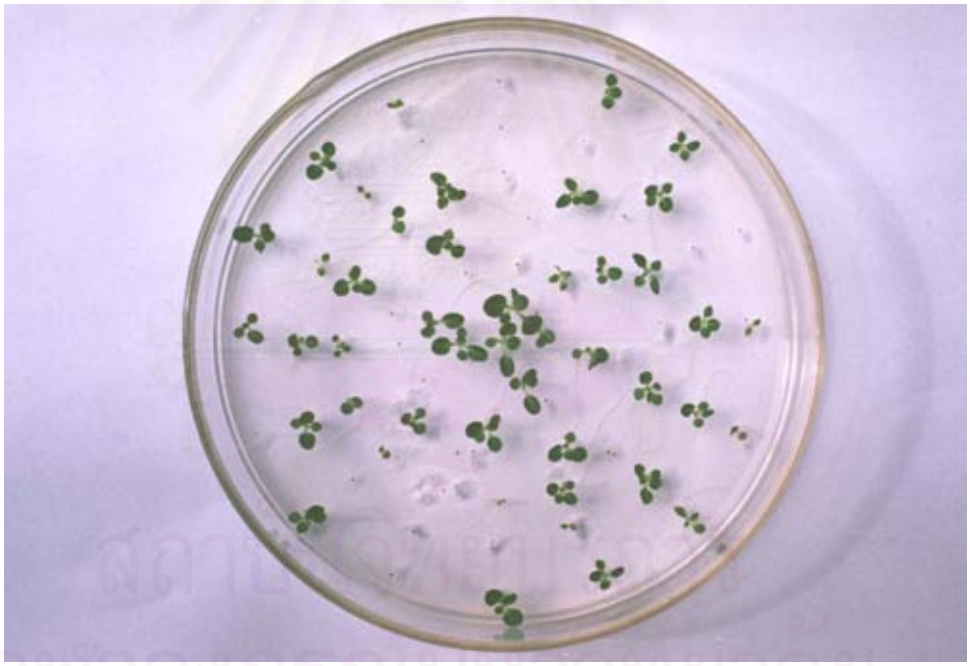


Figure 19 One-month-old of transgenic seedlings on MS medium, supplemented with 50 mg/L kanamycin.

The number of kanamycin resistant and sensitive seedlings were recorded. To determine how many insertions occurred in the transgenic lines, the Chi-square tests were performed based on the segregation of kanamycin resistant genes, which was used as the selectable marker gene for plant transformation. If the transgenic line contains one insertion, the progeny of T_1 transgenic line will show the kanamycin resistant trait segregation; kanamycin resistant: kanamycin sensitive = 3 : 1. The first hypothesis was proposed as the transgenic line had only one inserted T-DNA, which leaded to kanamycin resistant : kanamycin sensitive segregation at 3: 1 ratio. The probability in Chi-square test for the single insert in each transgenic lines was shown in Table 1. This hypothesis, was significantly accepted for lines T1, T2, T5, T6, T7, T8 and T9. Another Chi-square test for the hypothesis of two inserts of *TGG1* promoter ─ *gus* fused gene was performed. If the T_1 transgenic line has two inserted position in the genome. The kanamycin resistant trait segregation will show kanamycin resistant : kanamycin sensitive at 15:1 ratio. This hypothesis was tested for the transgenic lines, T3, T4 and T10, which results were shown in Table 2. All lines tested were performed similarly to the hypothesis.

Table 1 The Chi-square test of the transgenics having one insert of *TGG1*-promoter ─ *gus* fused gene.

P = 0.05 χ^2 = 3.84 df =1

Line	No. of planted seed	No. of resistant seeds	No. of sensitive seeds	$(O-E)^2/E^2$	Probability
1	52	33	4	3.340^{ns}	0.078
2	74	21	6	0.173^{ns}	0.740
3	50	37	1	3.929*	0.048
4	75	56	4	9.800*	<0.005
5	52	34	9	0.193^{ns}	0.720
6	54	39	10	0.333^{ns}	0.600
7	57	30	16	1.855^{ns}	0.190
8	121	68	14	2.341^{ns}	0.140
9	108	75	16	2.24^{ns}	0.150
10	50	36	5	4.694*	0.038

* = significantly different at P $\geq$ 0.05

ns = not significantly different

Table 2 The Chi-square test of the transgenics having two inserts of *TGG1*-promoter ─ *gus* fused gene

P = 0.05 χ^2 = 3.84 df =1

Line	No. of planted seed	No. of resistant seed	No. of sensitive seed	$(O-E)^2/E^2$	probability
3	50	37	1	1.974^{ns}	0.180
4	75	56	4	0.788^{ns}	0.410
10	50	36	5	0.654^{ns}	0.440

ns = not significantly different

4. Quantitation of *gus* gene expression regulated by *Arabidopsis TGG1* promoter in transgenic tobacco.

4.1 Gus gene expression regulated by *Arabidopsis TGG1* promoter in T_1 transgenic tobacco.

4.1.1 Expression in leaves

Leaf tissues at different ages were used for GUS activity analysis. GUS activity in leaf 1 ─ 12 (counted from the top) of soil grown plants were shown in table 3 and Figure 20. The GUS activity was ranging from 0.058 ─ 12.389 pmole MU/min/mg protein. The highest GUS activity was detected in the third leaf from the top.

Table 3 The GUS activity of the soil grown T_1 transgenic tobacco plant at different ages.

leaves position	GUS activity (pmol MU/min/mg protein)
1	12.000
2	9.083
3	12.389
4	10.981
5	4.522
6	3.249
7	0.330
8	0.058
9	0.044
10	0.129
11	0.201
12	0.250
Non-transformed leaf	0.011

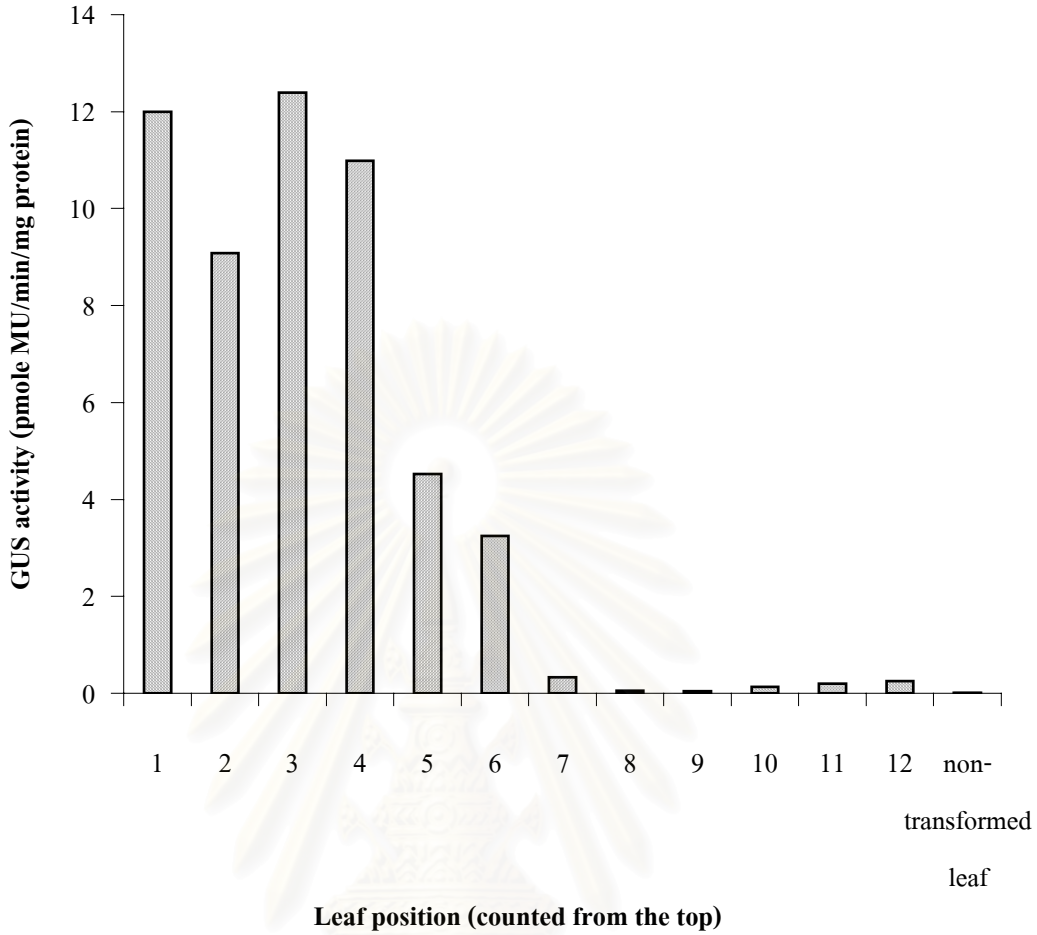


Figure 20 Comparison of GUS activity in leaves of different positions of T_1 transgenic tobacco.

4.1.2. Expression in reproductive organ and young fruit.

To determine GUS activity in floral organs during floral development, the floral bud or floral tissues were separated into 4 parts ; sepal, petal, stamen and pistil. Three developmental stages of floral organ were examined. The first stage was 4 days after floral bud formation (DAF). At this stage, the floral bud was 0.5 cm long and no pedicel formation. The second stage was 7 days after floral bud formation. At this stage the floral bud was 1.5 cm long with pedicel formation. The third stage, 10 days after floral bud formation, the flower was fully developed. It was the second day of floral opening, and anthesis occurred. After fertilization, which was the 15 days after floral bud formation, petal, stamen, stigma and style were senescing and detached from the flower. Ovary wall was developed into pericarp. At this stage, GUS activity analysis was performed on the tissues of immature fruit; sepal, pericarp, and immature seeds. GUS activity in different tissues of flowers during floral development was shown in Table 4 and Figure 21.

Table 4 The GUS activity in reproductive organ and immature fruit of T_1 transgenic tobacco.

type	organ	GUS activity (pmole MU/min/mg Protein)
4 DAF floral bud	sepal	12.14
	petal	7.33
	stamen	7.85
	pistil	8.47
7 DAF floral bud	sepal	8.10
	petal	1.44
	stamen	1.16
	pistil	1.06
	pedicel	16.22
10 DAF flower	sepal	12.16
	corolla tube	3.73
	corolla lobe	2.02
	anther	2.08
	filament	1.06
	stigma	2.06
	style	1.13
	ovary	0.07
	pedicel	27.16
15 DAF	sepal	10.89
immature fruit	seed	1.07
	pericarp	1.71
	pedicel	96.85
non transformed tissue		0.05

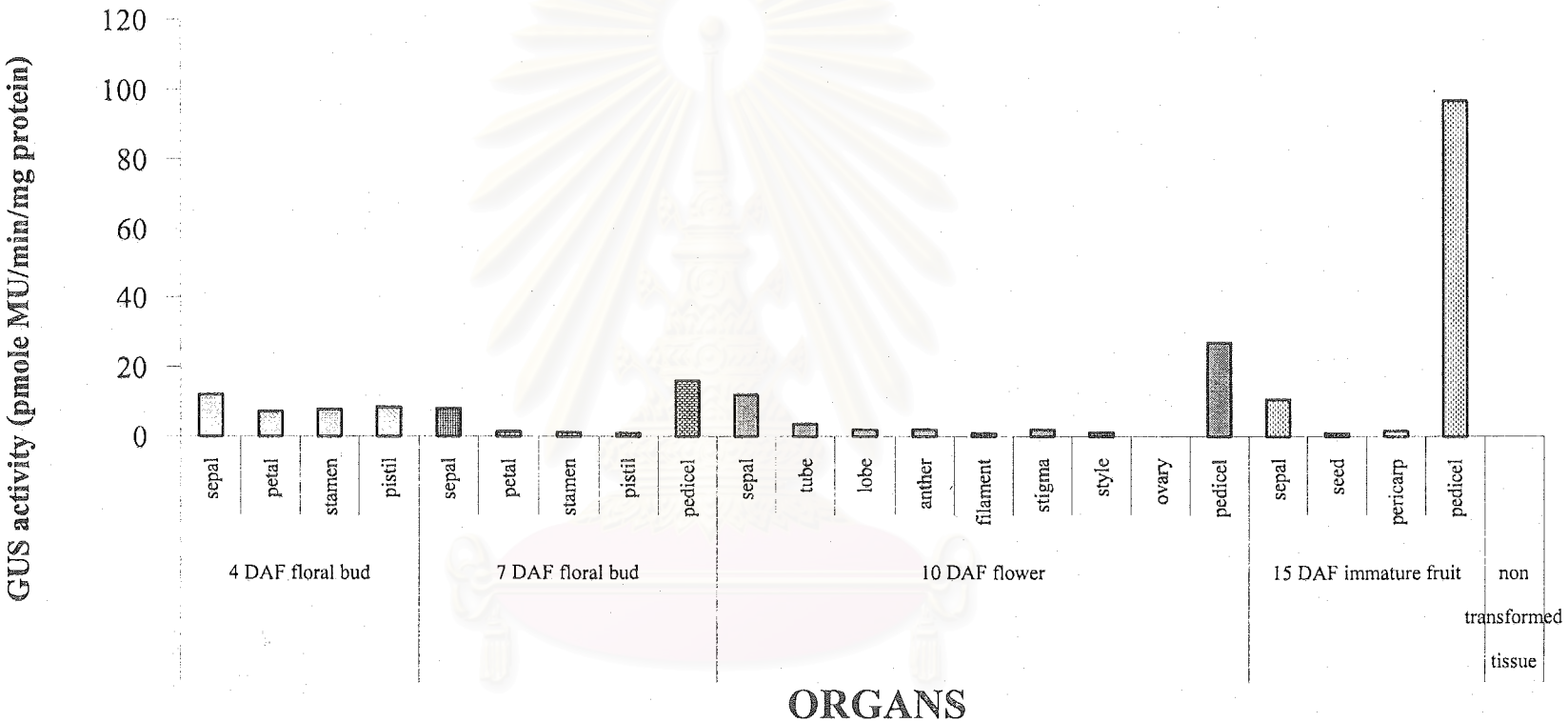


Figure 21 Comparison of GUS activity in different floral and immature fruit tissues of T_1 transgenic tobacco.

GUS activity was ranging from 7.33 ─ 12.13 pmole MU/min/mg protein in 4 DAF floral bud, 1.06 ─ 8.01 pmole MU/min/mg protein in 7 DAF floral bud, 0.66 ─ 12.16 pmole MU/min/mg protein in 10 DAF anthesis flower and 1.07 ─ 10.89 pmole MU/min/mg protein in developing fruit.

4.2 *Gus* gene expression regulated by *Arabidopsis TGG1* promoter in T_2 transgenic tobacco.

4.2.1 Expression in cotyledons

GUS activity was quantitated in cotyledons of the seedlings at different ages ; 3, 6, 9, 12 and 15 days after germination (DAG). The cotyledons were harvested from the kanamycin resistant seedlings, aseptically grown in the plant tissue culture room. The results was shown in Table 5 and Figure 22. GUS activity in cotyledons from 6, 9 and 12 DAG seedlings was significantly lower than the activity in the cotyledon of 15 DAG seedling with 95 % confident level.

Table 5 Comparison of GUS activity in T_2 transgenic tobacco cotyledons at different ages.

Age of Cotyledon (day)	GUS activity (pmole MU/min/mg protein) mean*	max	min	+SD
3	170.362	198.840	140.040	31.275
6	142.979	158.120	130.750	14.160
9	118.6145	139.000	96.630	17.390
12	128.057	175.150	99.905	35.997
15	204.409	270.730	151.730	51.124

* = significantly different at 95% confident level

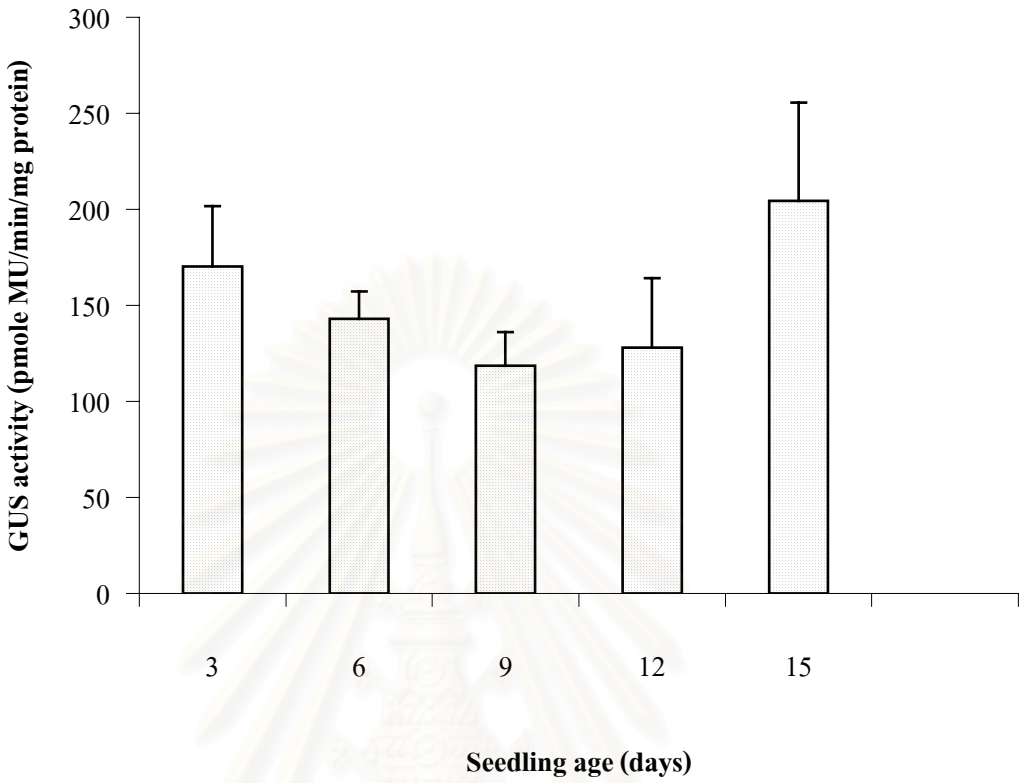


Figure 22 Comparison of the GUS activity in cotyledons of transgenic tobacco, cultured *in vitro*.

4.2.2 Expression in leaves

Leaf tissues at different ages were used for GUS activity analysis. The studies were separated into two experiments. One was performed with the 8 week-old aseptically grown plants, while the other was done with the same age, soil grown plants. The leaf tissue from non ─ transformed tobacco was used as the negative control.

The 8 week-old aseptically grown tobacco plants developed more leaves than the soil-grown plants. Therefore, fourteen leaves were used for GUS activity quantitation. The result of GUS activity in different leaf position was shown in table 6 and figure23. The range of GUS activities was 10.97 ─ 48.54 pmole MU/min/mg protein. . The highest activity was detected in the fifth leaf from the top, which was significantly higher than the activity in leaf 10 ─ 14 at 99 % confident level.

Table 6 Comparison of GUS activity in leaves at different positions of aseptically grown T_2 transgenic tobacco.

	GUS activity (pmole MU/min/mg protein)			
Leave position	mean**	max	min	+SD
1	28.958	51.769	7.557	19.286
2	40.922	65.444	21.710	21.992
3	30.917	36.132	21.146	6.5144
4	37.068	46.462	28.133	9.352
5	48.536	77.305	36.821	19.308
6	45.699	77.903	21.324	23.538
7	40.970	50.184	18	15.079
8	36.214	56.296	538	17.043
9	28.372	45.381	16.883	13.565
10	17.096	24.184	10.689	6.777
11	10.965	17.576	8.014	4.633
12	11.907	16.593	6.779	4.275
13	14.089	19.948	9.886	3.004
14	16.151	22.625	10.643	4.940
Non transformed leaf	0.366	0.368	0.364	1.85E-17

** = significantly different at 99% confident level

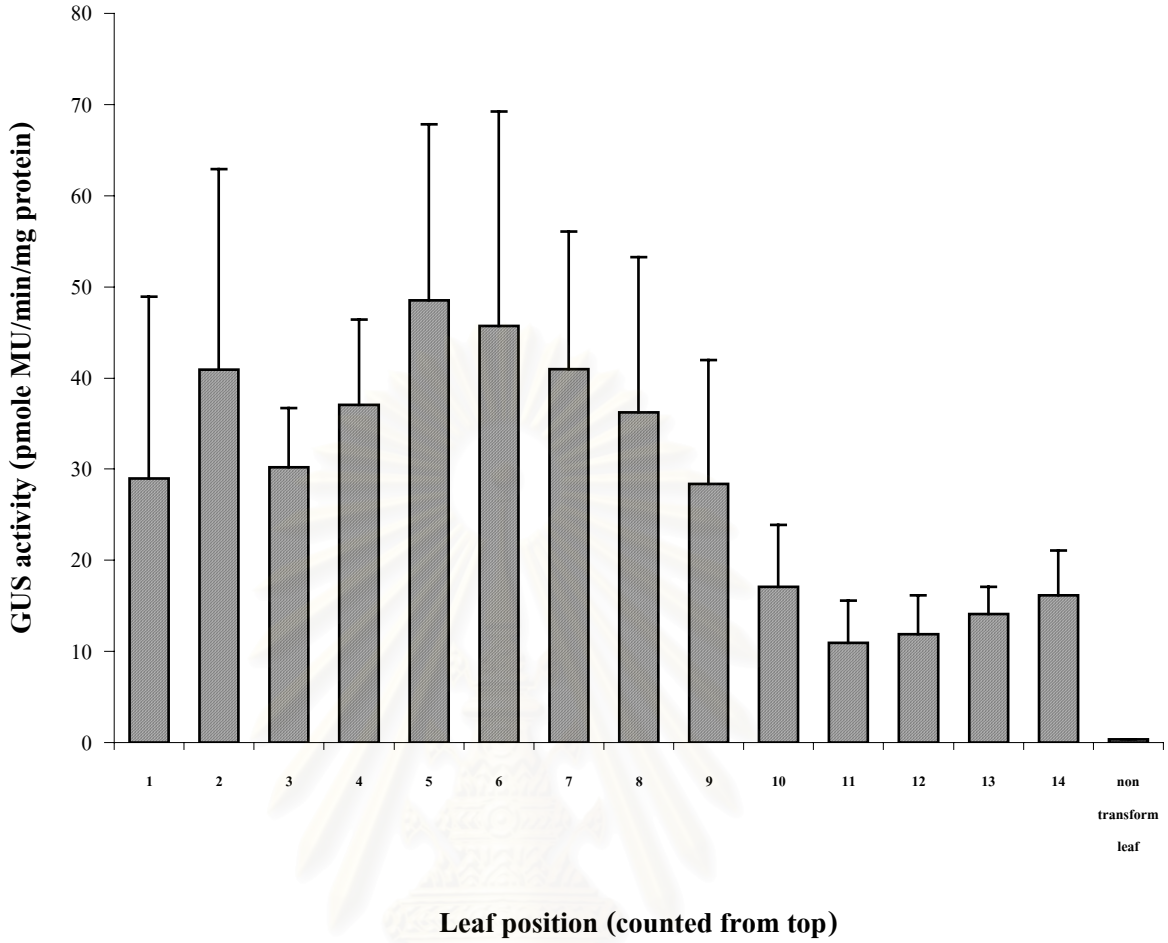


Figure 23 Comparison of the GUS activity in leaves 1-14 of the 8- week-old aseptically grown T_2transgenic tobacco plants.

GUS activity in leaf 1 - 12 (counted from the top) of the soil grown plants wes shown in figure24. The GUS activity was ranging from 0.48 ─ 5.12 pmole MU/min/mg protein, this result was shown in table 7. The highest GUS activity was detected in the second leaf from the top, which was significantly higher than GUS activity in the lower leaves; leaf 7 ─ 12 at 99 % confident level.

Table 7 Comparison of GUS activity in leaves at different positions of soil grown T_2 transgenic tobacco.

	GUS activity (pmole MU/min/mg protein)			
Leave position	mean**	max	min	+SD
1	2.752	4.541	0.889	1.907
2	5.117	10.889	1.926	4.219
3	2.254	2.803	1.460	0.595
4	2.087	2.481	1.620	0.417
5	2.733	2.920	2.650	0.127
6	3.046	4.991	1.113	2.1631
7	1.492	2.610	0.438	1.187
8	0.955	1.685	0.202	0.816
9	0.482	0.829	0.176	0.348
10	0.766	1.272	0.489	0.369
11	0.348	0.419	0.279	7.83E-2
12	0.616	0.967	0.276	0.375
Non transformed leaf	1.11E-02	1.11E-02	1.10E-02	5.78E-19

** = significantly different at 99% confident level

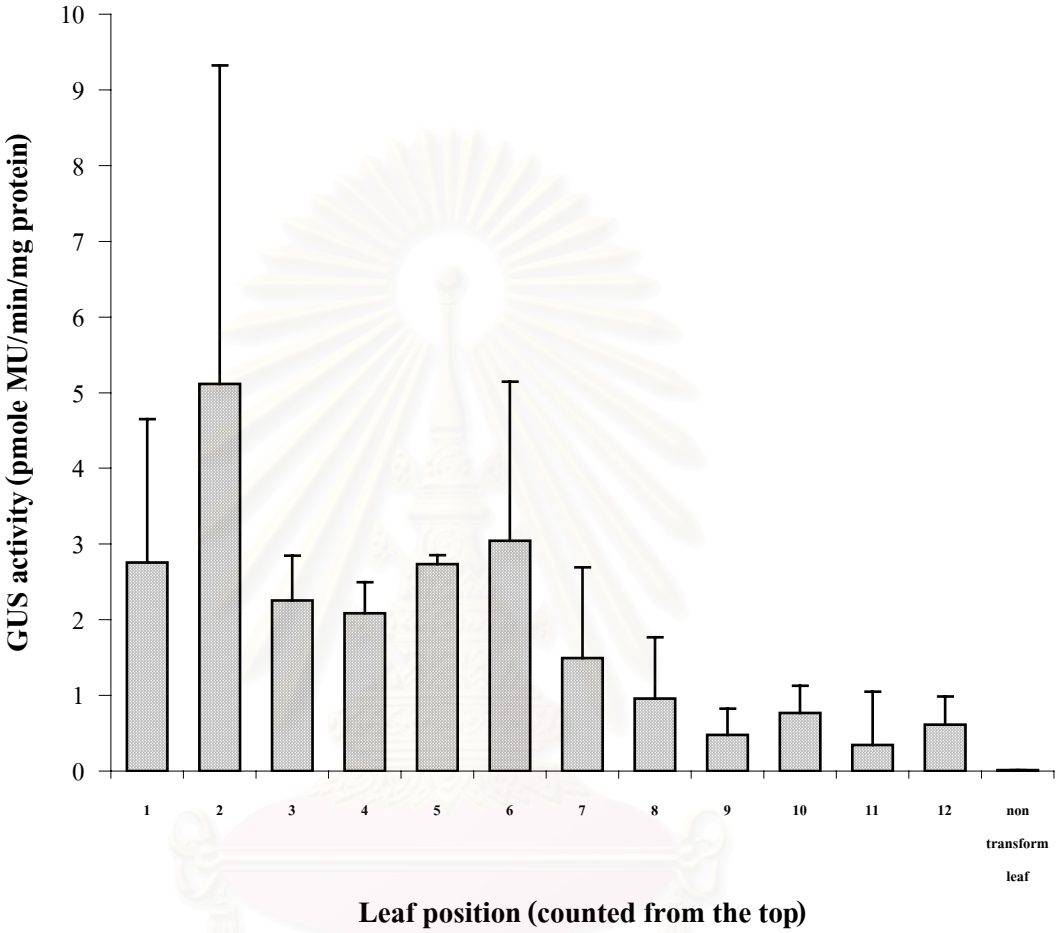


Figure 24 Comparison of the GUS activity in leaves at different position of the 8 week-old soil grown T_2 transgenic tobacco plants.

4.2.3. Expression in reproductive organs and young fruit of T_2 tobacco plants.

To determine GUS activity in floral organs during floral development, the floral buds and flowers were detached into 4 parts; sepal, petal, stamen and pistil. Three developmental stages of floral organs and developing fruit, as described earlier, were studied. GUS activity in different whorls of flowers during floral development was shown in Table 8 and Figure 25.

GUS activity was ranging from 13.14 ─ 28.85 pmole MU/min/mg protein in 4 DAF floral bud, 0.96 ─ 4.51 pmole MU/min/mg protein in 7 DAF floral bud, 0.05 ─ 39.66 pmole MU/min/mg protein in 10 DAF anthesis flower and 0.11 ─ 16.87 pmole MU/min/mg protein in developing fruit.

Table 8 The GUS activity in reproductive organ and immature fruit of T_2 transgenic tobacco.

Type	organ	GUS activity (pmole MU/min/mg Protein)
4 DAF floral bud	sepal	28.85
	petal	17.77
	stamen	13.14
	pistil	15.21
7 DAF floral bud	sepal	4.51
	petal	1.11
	stamen	1.33
	pistil	0.96
	pedicel	4.51
10 DAF flower	sepal	12.04
	corolla tube	3.96
	corolla lobe	3.24
	anther	14.32
	filament	1.63
	stigma	1.70
	style	1.71
	ovary	0.05
	pedicel	39.66
15 DAF	sepal	0.11
Immature fruit	seed	1.78
	pericarp	16.87
	pedicel	0.05
non transformed leaf		91.37

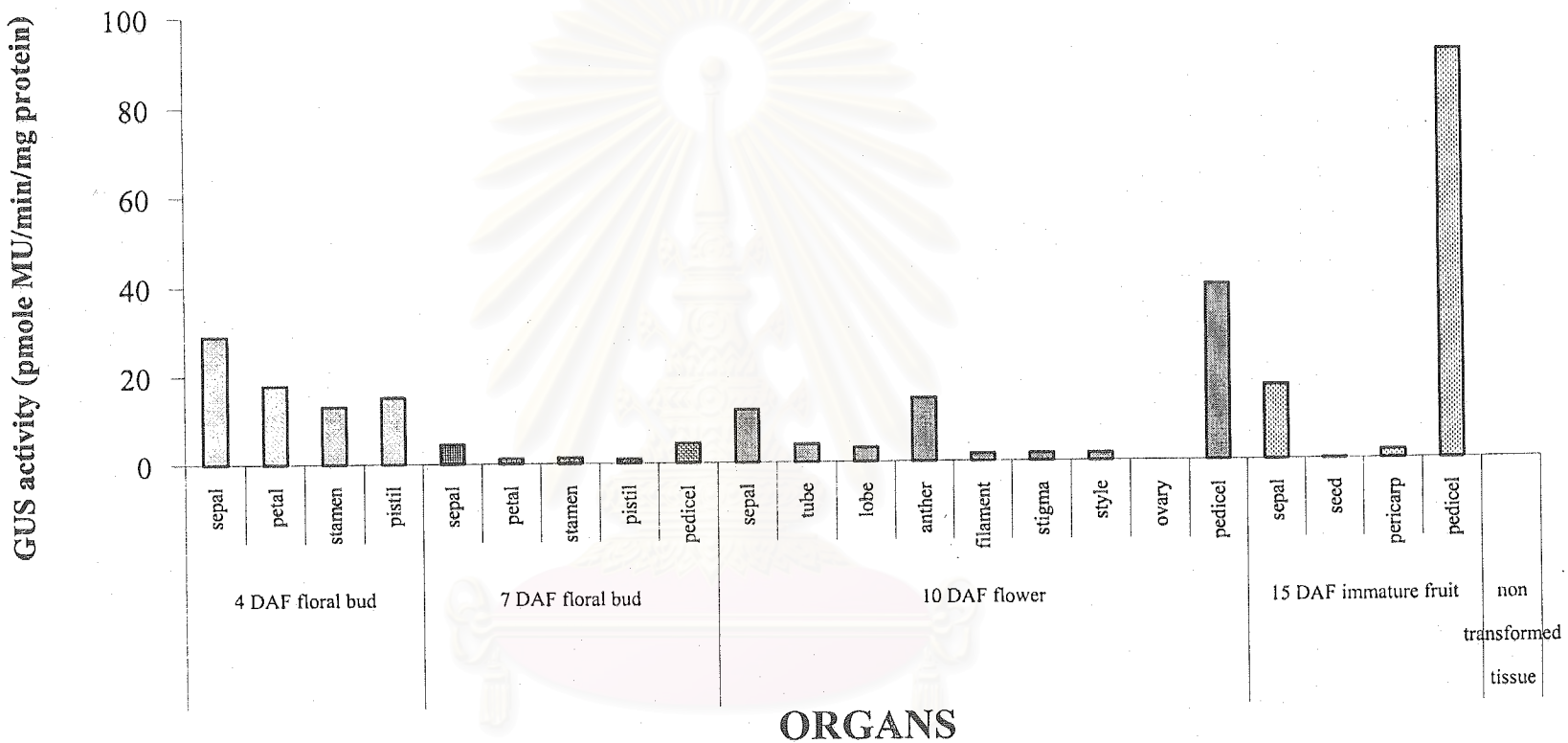


Figure 25 Comparison of GUS activity in different floral and immature fruit tissues of T_2 transgenic tobacco.

5. Histochemical localization of GUS enzyme under *Arabidopsis TGG1* promoter regulation in transgenic tobacco.

GUS analysis was done on vegetative and reproductive organs of primary transgenic (T_1) and self-fertilized progeny (T_2) of the primary transgenic tobacco. For histochemical analysis of *gus* expression, whole seedlings, cross-section and long-sections of different organs of plant tissuses were submerged in staining solution (Stomp, 1992). Ten independent transgenic plants were assayed histochemically for GUS activity. All transgenic lines exhibited the same qualitative expression pattern, although the expression levels were variable.

In leaves of T_1 (Figure 26 b, c) and stems (Figure 27 a), *gus* expression was also mostly localized with in guard cells. No GUS expression was present in roots at any stage of development (Figure 27 b). The non-transformed leaf showed no *gus* expression (Figure 27 d)

Flowering organs of transgenic tobacco plants exhibited GUS activity in similar pattern observed in leaves. Guard cells of different floral organs, sepal, petal, anther, style and epidermal layer of ovary, as well as guard cells on pedicel, show GUS activity (Figure 28 a-c, Figure 29 a-d and Figure 27 c). Some pollens did contain GUS activity (Figure 29 e). No GUS enzyme activity was observed in stigma tissue and there were same in the guard cell of filament.

GUS expression in the T_2 tobacco lines was similar to what found in T_1 plants. The GUS activity in young leaves seemed to be very high, showing no specific localization in guard cell (Fig 26 a). But *gus* expression was mostly localized within guard cells of cotyledon and older leaves. However, at later stage the expression was more localized in guard cells of all tissues, except pollen, which had no gurad cell, but some of which contained GUS activity (Fig 29 e).

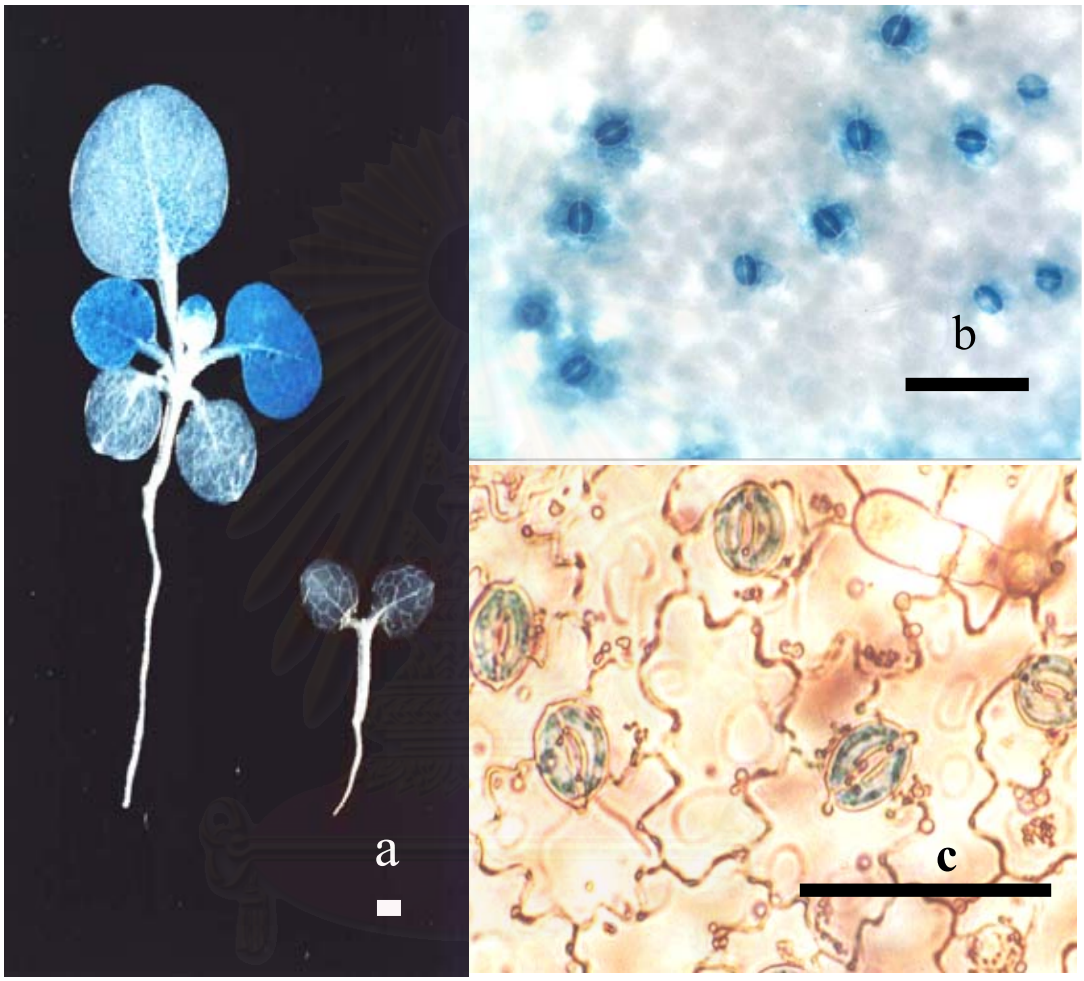


Figure 26 Histochemical assay of GUS enzyme in cotyledon and leaf tissues of transgenic tobacco plants (bar = 50 μ)

a. : Kanamycin resistant and sensitive transgenic tobacco seedling.

b. : Guard cells on leaf of transgenic tobacco seedling.

c. : Guard cells on epidermal peel of young transgenic tobacco leaf.

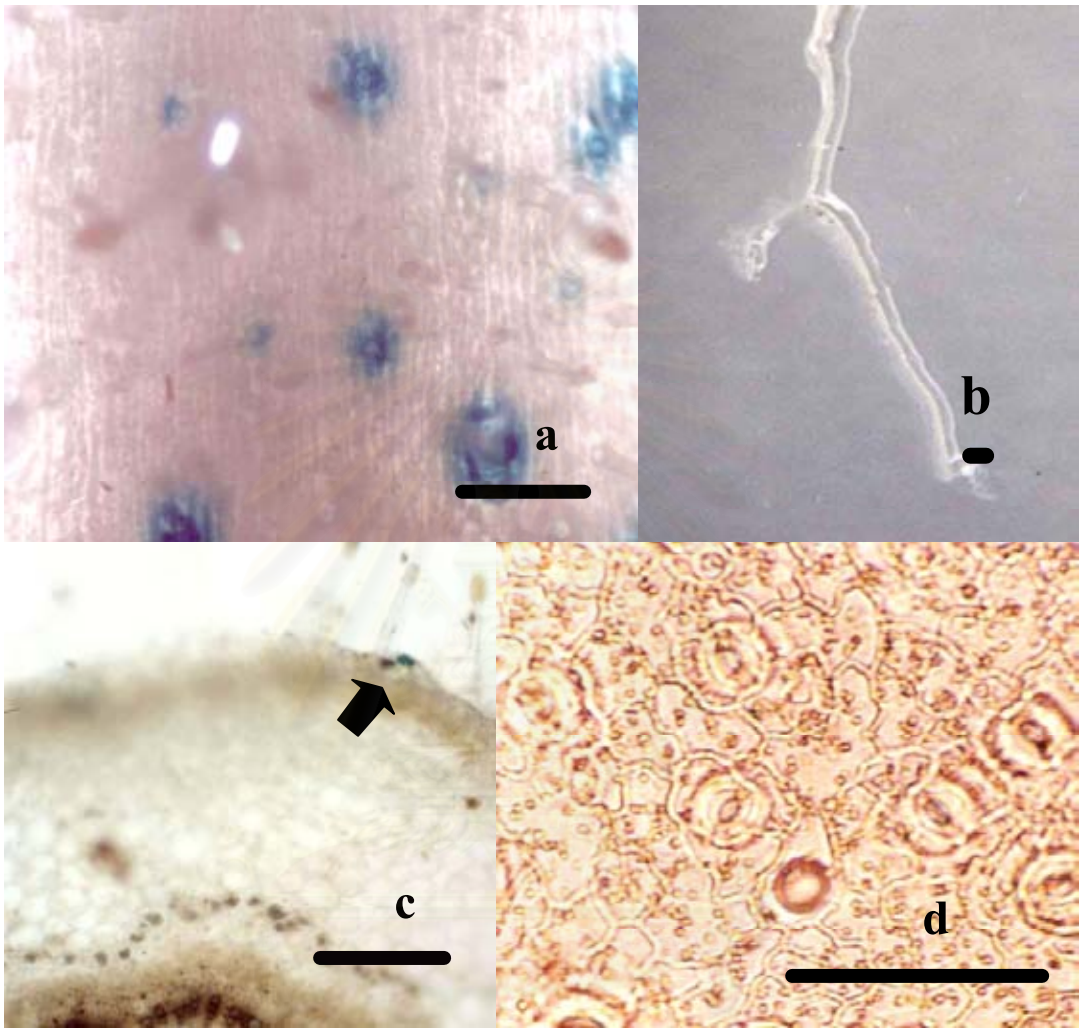


Figure 27 Histochemical assay of GUS enzyme in stem, root, and pedicel tissues of transgenic tobacco plants (bar = 50 μ)

a. : Guard cells on stem of transgenic tobacco.

b. : Root of transgenic tobacco.

c. : Cross-section of pedicel of transgenic tobacco.

d. : Guard cells on epidermal peel of non-transformed tobacco leaf.

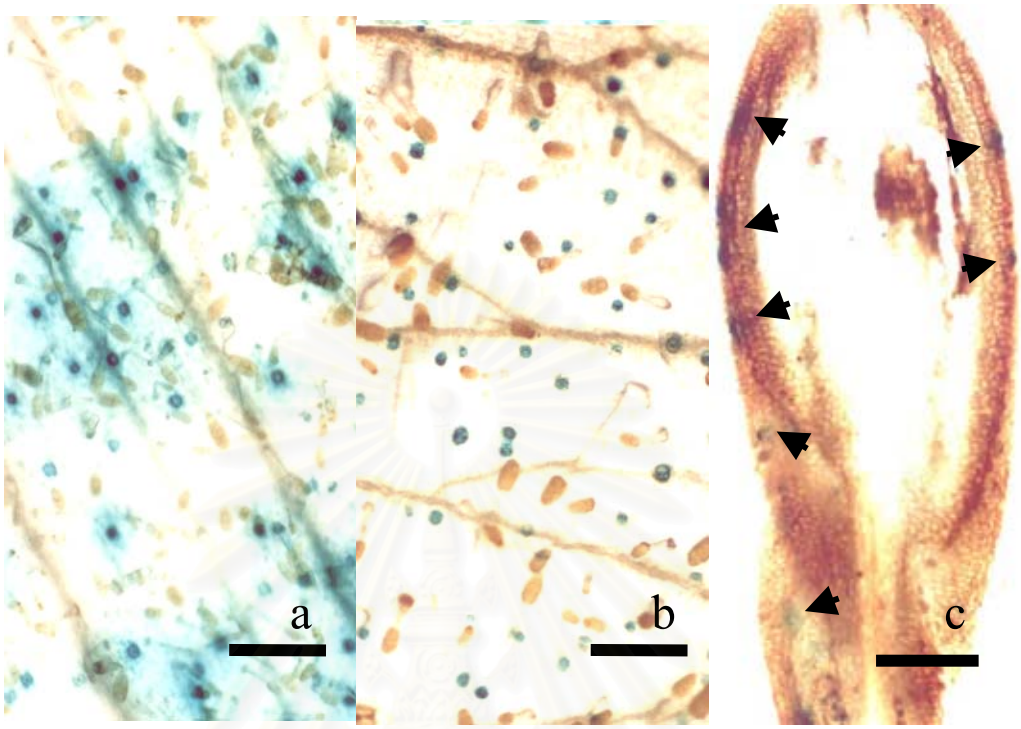


Figure 28 Histochemical assay of GUS enzyme in sepal, petal and anther of transgenic tobacco plants. (bar = 50 μ)

a. : Guard cells on corolla tube tissue of of transgenic tobacco.

b. : Guard cells on corolla lobe tissue of transgenic tobacco.

c. : Long -section of anther of transgenic tobacco.

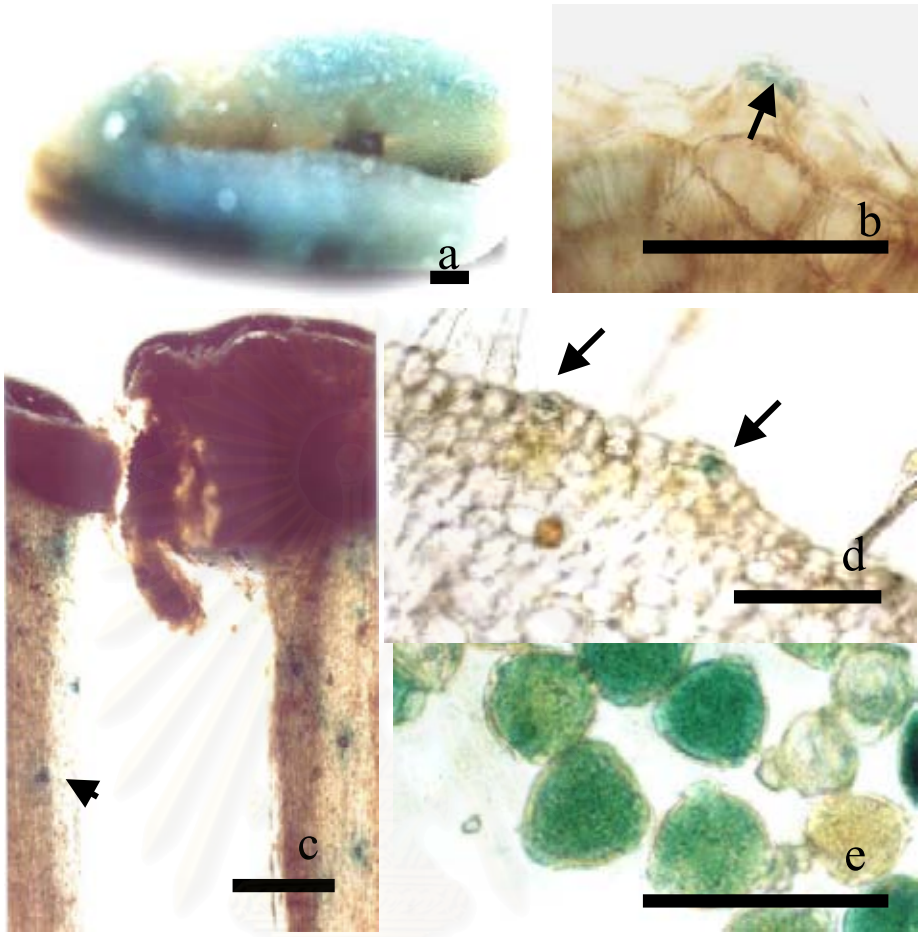


Figure 29 Histochemical assay of GUS enzyme in anther and pistil of transgenic tobacco plants. (bar = 50 μ)

a. : Anther of transgenic tobacco.

b. : Cross -section of anther of transgenic tobacco.

c. : Stigma and style of transgenic tobacco.

d. : Cross-section of ovary of transgenic tobacco.

e. : Mature pollens of transgenic tobacco.

6. Quantitation of *gus* gene expression regulated by *Arabidopsis TGG1* promoter in transgenic petunia.

GUS activity were fluorometrically assayed (Gallagher, 1992) in the total protein extract from the third leaf from the top and various tissues of 5 DAF flower; pedicel, sepal, petal, stamen and pistil. The GUS activity in different tissues of the soil grown transgenic petunia showed the significant difference at 99 % confident level. This result was shown in Table 9 and figure 30. The GUS activity was ranging from 0.89 ─ 5.73 pmole MU/min/mg protein. The GUS activity in the transgenic petunia was higher than that detected in the non-transformed petunia leaf tissue. Means of GUS activity, the GUS activity in stamen, pistil and pedicel were similar, but significantly higher than GUS activity in young leaf, sepal and petal.

Table 9 Comparison of GUS activity in different tissues of transgenic petunia plants.

	GUS activity (pmole MU/min/mg protein)			
ORGANS	mean**	max	min	+SD
Young leaf	1.211	1.497	0.755	0.355
Sepal	0.895	1.105	0.512	0.278
Petal	1.081	1.175	0.951	0.170
Stamen	3.528	5.852	2.086	1.621
pistil	5.726	6.098	5.048	0.478
pedicel	3.617	6.929	1.989	2.284
Non transformed tissue	0.338	0.338	0.338	9.25E-12

** = significant at 99% confident level

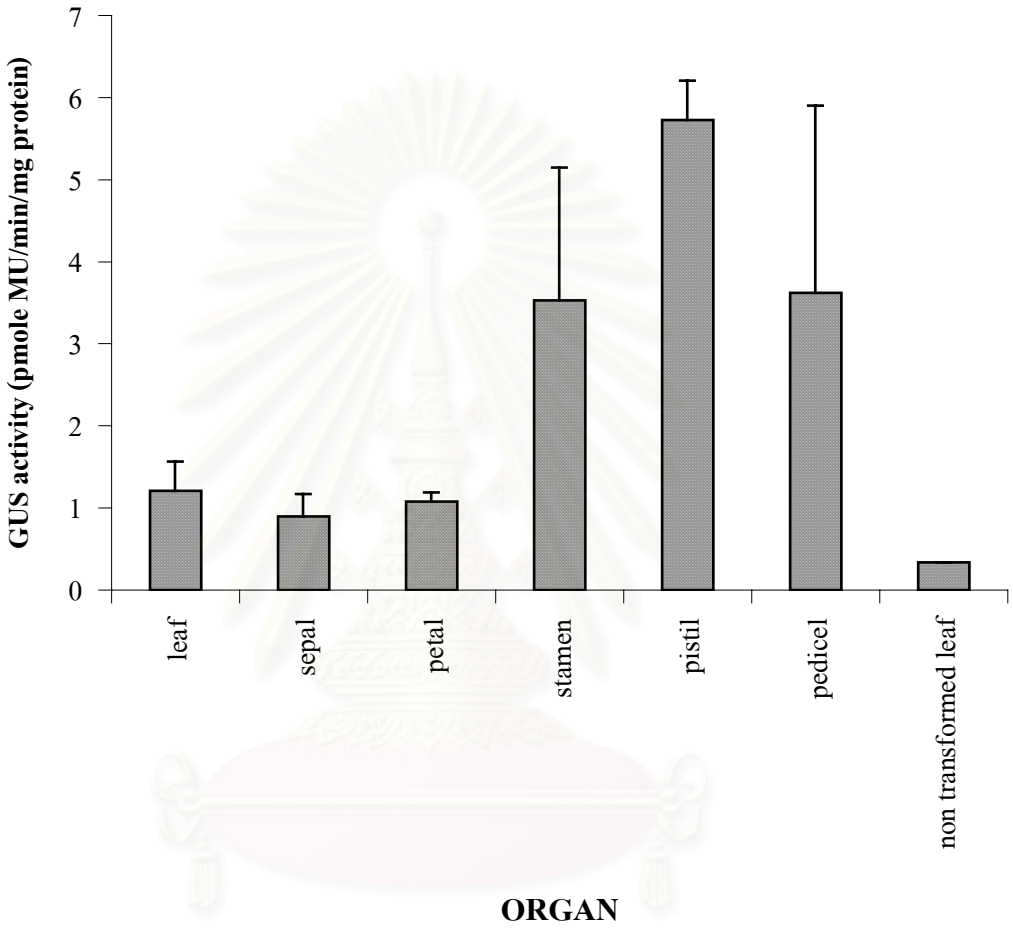


Figure 30 Comparison of GUS activity levels in different tissues of transgenic petunia.

7. Histochemical localization GUS enzyme under *Arabidopsis TGG1* promoter regulation in transgenic petunia.

GUS analysis was done on vegetative and reproductive organs of primary transgenic petunia. For histochemical analysis of *gus* expression, epidermal peel and whole of different organs of plant tissuses were submerged in the staining solution (Stomp, 1992). For vegetative organs, the GUS enzyme was clearly localized in guard cells of young leaf and petiole (Figure 31 a and d).

In different floral organs, sepal, petal, anther, and pollen showed GUS activity (Figure 31 b, c and e). No GUS enzyme activity was observed in pistil.

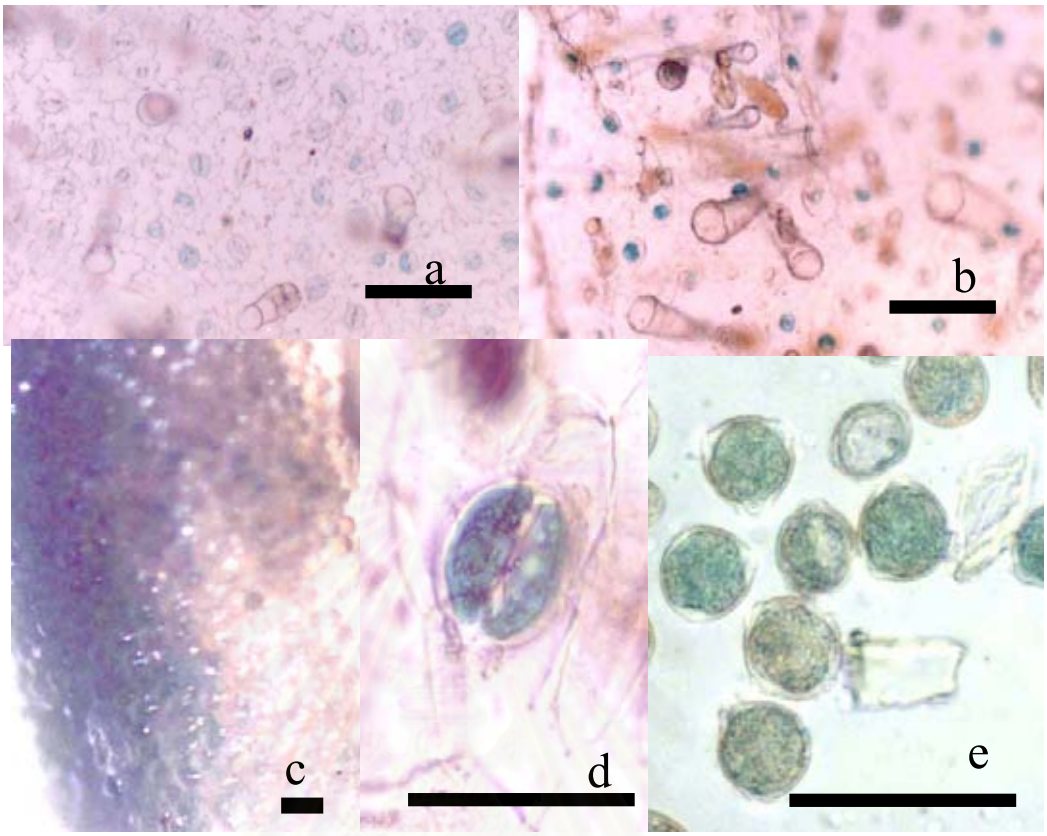


Figure 31 Histochemical assay of GUS enzyme in different tissues of transgenic petunia plants (bar = 50 μ)

a. : Guard cells on epidermal peel of young transgenic petunia leaf.

b. : Guard cells on sepal of young transgenic petunia flower.

c. : Anther of young transgenic petunia.

d. : Guard cells on petiole of young transgenic petunia.

e. : Pollen of young transgenic petunia.

8. Quantitation of *gus* gene expression regulated by *Arabidopsis TGG1* promoter in transgenic potato.

GUS activity was fluorometrically assayed in leaf and stem extract of all independent lines of one-month-old transgenic potato plants. The results was shown in Table 10 and Figure 32. GUS activity could be detected in all independent transgenic potato lines, while the non-transformed plant had very low GUS activity. The highest GUS activity was found in Pro A Pz6.

Table 10 The GUS activity in nine lines of independent transgenic potato.

Transgenic potato lines	GUS activity (pmole MU/min/mg protein)
ProA Pzn 1	2.125
ProA Pzn 2	1.047
ProA Pzn 3	1.407
ProA Pz 1	1.161
ProA Pz 2	0.813
ProA Pz 3	0.931
ProA Pz 4	1.326
ProA Pz 5	2.099
ProA Pz 6	2.356
Non-transformed plant	0.114

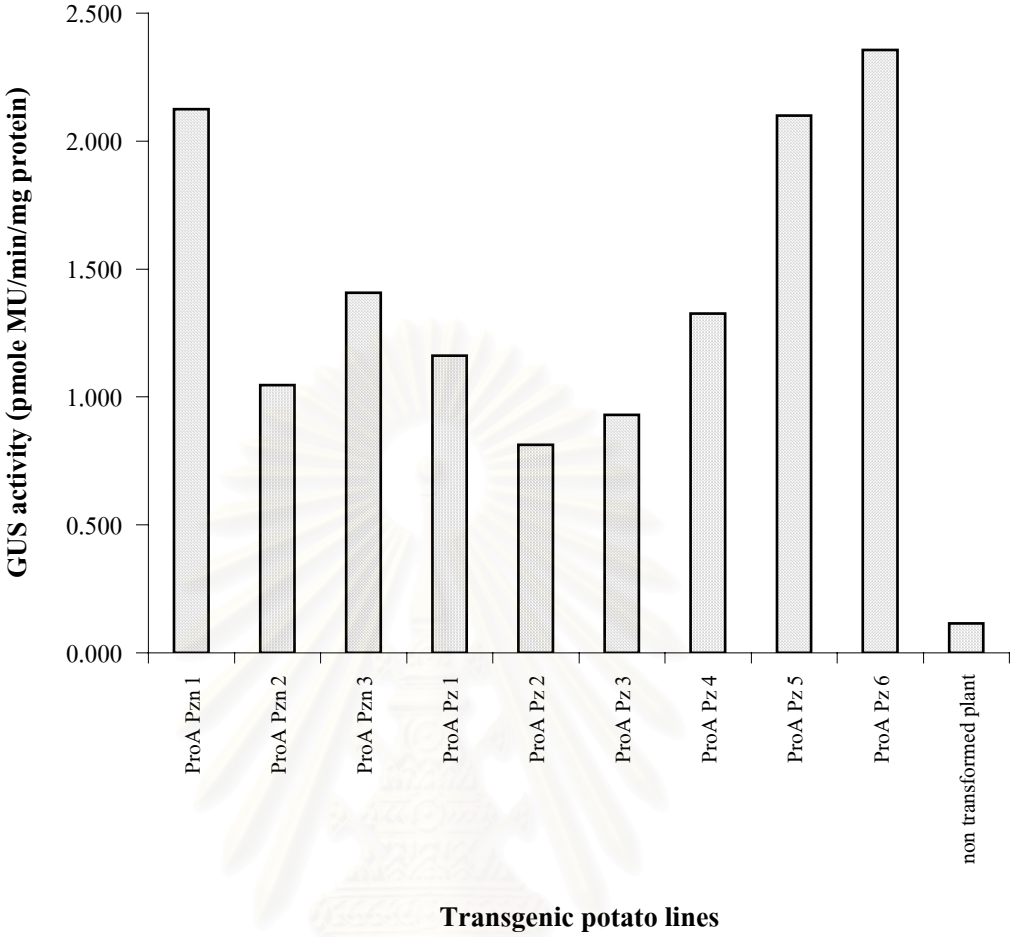


Figure 32 Comparison of GUS activity in nine lines of independent transgenic potato, cultured *in vitro*.

9. Histochemical localization of GUS enzyme under *Arabidopsis TGG1* promoter regulation in transgenic potato.

GUS analysis was done on vegetative parts of primary transgenic potato, maintained *in vitro*. For histochemical analysis of *gus* expression tissue of leaf and stem were submerged in staining solution (Stomp, 1992) and incubated for one days. No GUS activity could be detected in any tissues (Figure 33).

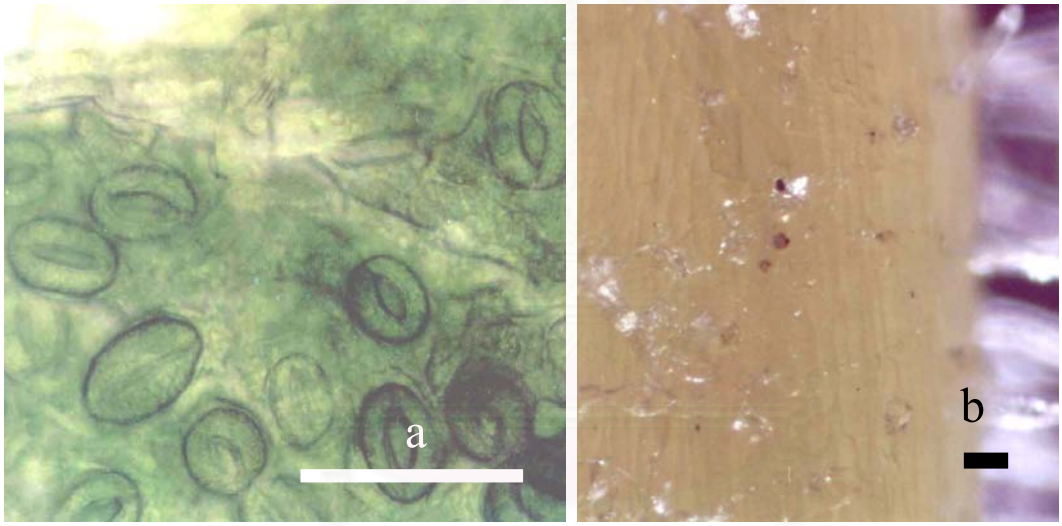


Figure 33 Histochemical assay of GUS enzyme in leaf (a) and stem (b) of transgenic potato plants.

CHAPTER V

DISCUSSION

I Growth curve of *A. tumefaciens* LBA4404-ProA1.1

Growth curve of *A. tumefaciens* LBA4404-ProA1.1 grown in liquid Mg/L medium exhibited a normal pattern of bacterial growth curve (McKance and Kandel, 1996). It showed distinct phases of growth : (1) lag phase, (2) logarithmic phase and (3) stationary phase. Normally, growth curve consists of four different phases ; lag phase, logarithmic phase, stationary phase and death phase. However, in this experiment, the purpose was to determine the appropriate time for cell harvest at late log phase to obtain the proper cells for plant transformation. Therefore, the experiment was terminated before the *Agrobacterium* culture turned into death phase.

Based on the growth curve of *A. tumefaciens* LBA-ProA1.1 in Mg/L liquid medium, 6-12 hours after inoculation exhibited the logarithmic phase pattern, and after 12 hours, the stationary phase was reached. It was noticed that the time interval for measuring cell population sizes was too long to determine the exact turning point for each phase. However, the result was good enough to determine the appropriate cell harvest time for plant transformation process. Several reports (Feldmann, 1995; Dietze *et al*, 1995 refered by Potrykus and Spangenbag, 1995) suggested to use *Agrobacterium* cells in the late log phase to stationary phase for plant transformation. Therefore, *Agrobacterium* used for all plant transformation experiment was harvested during 12-16 hours after inoculation of 1 ml of overnight grown culture.

Several medium recipes, LB (Horsh *et al*, 1985), YEB (Dietze *et al*, 1995, cited by Potrykus and Spangenbag, 1995), and MYA (Filho *et al*, 1994) were used for *Agrobacterium* growth for plant transformation. Lin *et al* (1994) indicated the value of

OD_{620} for measuring cell concentration of *Agrobacterium* in LB, OD_{620} = 1 determines 5 x 10^8 cells/ml. However, in my experiment, when OD_{620} is equal to 1, *A. tumefaciens*, LBA4404-ProA1.1 cells concentration is equal to 5 x 10^{11} cells/ml. The difference in the relationship between the optical density value at 620 nm and cell concentration may be caused by the stage of the cell, cell secretion, and cell morphology when they were grown in different medium.

II Solanaceous plant transformation

Solanaceous plants were used as the model plants for the experiments, according to their characters for not having the natural myrosinase-glucosinolate system, and availability in the transformation methodology for these plant species (McCormick *et al*, 1986; Leij *et al*, 1991; Gama, 1996)

Agrobacterium cell suspension for plant transformation was done according to Lin *et al* (1994), while the co-cultivation and selection for transgenic plant methods were varied depending on plant species. For tobacco transformation, the transformation process was done by following the protocol reported by Lin *et al* (1994), except that the Mg/L medium was used instead of LB medium. The efficiency for tobacco plant transformation was about 72%, which were comparable to Lin *et al* (1994).

For petunia transformation, *Agrobacterium* transformation was done in the same procedure, but the preculture treatment co-cultivation and transgenic plant selection were done according to Smith (1992). Up to 85% efficiency was obtained in petunia transformation. However, due to the fungal infestation during transformation process, and the sensitivity of petunia plants in transferring to the soil grown condition, the number of transgenic plants obtained were very low. To extend the period of acclimatization before transplanting may help solving the latter problem.

Several protocols for genetic transformation using leaf discs and potato tuber discs have been successfully employed to generate transgenic plants (Stiekema *et al*, 1988; Imai *et al*, 1993; Benchekroun *et al*, 1995; Filho *et al*, 1994; Beaujean *et al*, 1998). However, all these procedures were not applicable for potato, cultivar Spunta, which is the commercial potato cultivar in Thailand. Thanyarat (1999) developed two medium recipes for *Solanum tuberosum* L. cultivar Spunta internodal regeneration; MS complete medium, supplemented 1.0 mg/L Zeatin and 0.01 mg/L NAA; and MS complete medium supplemented with 1.0 mg/L Zeatin. The regeneration time was about 8-10 weeks. The two recipes then were tested for transformation application by using as the preculture medium, co-cultivation medium and regeneration medium. The transgenic shoots were obtained in 4 weeks after co-cultivation process. However, the efficiency of potato cultivar Spunta transformation was still very low, less than 10%, when compared to the transformation efficiency in potato cultivar Desiree, Bintje and Kaptah Vandel, transformed with Beaujean *et al*'s method (1998). However, their method was tried by Kritapong (1997), but it was shown that it was not applicable for potato cultivar Spunta.

III Determination of the inserted fragment numbers and independence of the tobacco transgenic lines.

To prove the transgenic line independence was required for all investigation using reporter gene system to determine promoter regulation. Five to ten independent lines, showing the consistant pattern of gene expression, are considered to be the reasonable representation of the promoter regulation.

In this study, two methods were used to show that ten transgenic tobacco lines used in this study were independent. The genomic Southern Blot of all line using the linearized ProA1.1 plasmid as a probe, showed the approximately identical 3 kb positive band. This positive 3kb fragment was expected. When digested with HindIII

and *Eco*RI genomic DNA from all transgenic lines, which contained the T-DNA region from ProA1.1 plasmid, should release the 3 kb HindIII ─*Eco*RI fragment (see ProA1.1 map in Figure 8). The other positive fragments refered to the positive region that could hybridize with NPTII gene, regulated with CaMV35S promoter and nos-terminator. The size of this region would be variable, depending on the position of insertion in the genome. Therefore, the variable in the sizes of positive region in transgenic lines indicated the transgenic independence.

Moreover, the numbers of positive fragment (besides 3 kb fragment) would indicate the numbers of insertion. Therefore, based on the Southern Blot analysis, tobacco line T4 and T10 were the only two lines, having T-DNA inserted into two region in the genome. These results were consistent with the segregation of kanamycin resistant trait in T_2 seedlings. However, there is an ambiguous result in T3 transgenic line. Based on the Chi-square test for a single insertion, the hypothesis was rejected. Contrary to the Southern blot analysis, it was shown that there were only two positive fragment sizes, 3 kb and 4.5 kb fragment.

To determine how many inserted region occurred in this transgenic line, the larger population size of the T_2 seeds should be done for segregation of kanamycin resistant trait analysis in T_2 seedlings. If the same result is confirmed, it will indicate that the NPTII positive fragments in this line are released in the 4.5 kb and 3.0 kb fragments, which is located in the same position as the positive signal of *Hin*dIII-*Eco*RI reporter gene fragment.

IV *Arabidopsis TGG1* promoter - *gus* gene expression in transgenic tobacco.

TGG1 promoter ─ *gus* gene expression showed age-dependent character.

Quantitative analysis of GUS activity was done in different aged leaf tissues. Both T_1 and T_2 soil grown transgenic plants showed the similar results (Figure 20 and 24). The younger leaves had higher level of GUS activity than the older leaves. Leaves from the *in vitro* culture T_2 transgenic lines showed the consistent result on age dependent expression. However, much higher GUS activity was detected in the approximately the same aged leaves (Figure34). It suggested that the environmental condition should effect *TGG1* promoter expression.

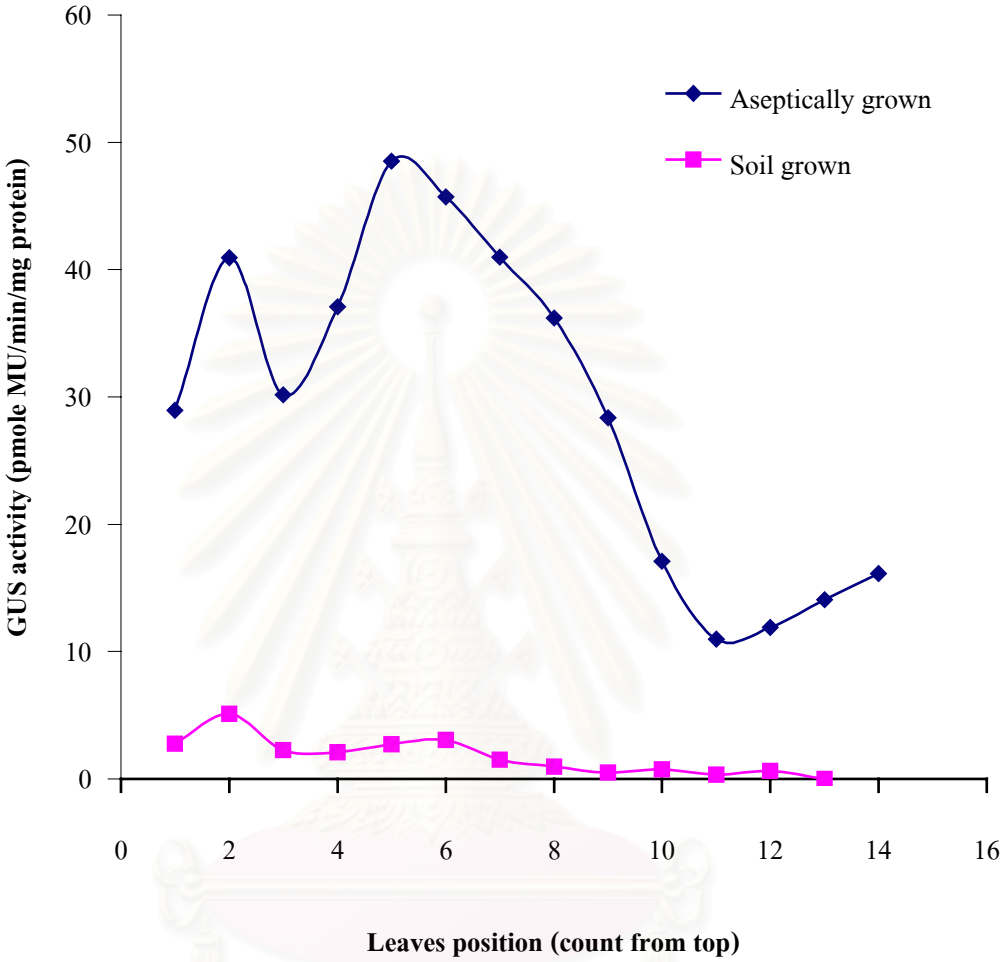


Figure 34 Comparison of the GUS activity in leaves of soil grown and aseptically grown transgenic tobacco.

High GUS activity was detected in cotyledons of the T_2 seedlings. Age dependent tendency of *TGG1* gene expression was also noticed in cotyledon tissue. However, the time course for GUS activity analysis was not long enough to clearly indicated the decreased GUS activity in older cotyledons.

The age dependent of *TGG1* gene expression was similar to what found in other species; *Arabidopsis thaliana* (Chadchawan, 1995) ; *Sinapis alba* (Pihakaski and Pihakaski, 1978)

Age ─ dependent character of TGG1 promoter was also detected in floral organs
of transgenic tobacco. In floral bud stage, the younger tissues (4DAF) had higher level of GUS activity than the older ones (7DAF). However, when the flower was open, and anther occurred, the *TGG1* promoter ─ *gus* gene expression was increased (Figure 26), especially in pedicel, and sepal tissues. Even more GUS activity was detected in sepal and pedicel of developing fruit. This data supported the plant-defense role of *TGG1* gene in Brassicaceous plant species (Iversen and Baggerud, 1980)

Age dependence of *TGG1* gene expression in floral tissues was consistent in both T_1 and T_2 transgenic tobacco plants (Figure 21 and 25).

V Guard cell specific expression of *Arabidopsis TGG1* promoter in trangenic tobacco.

Gus gene expression regulated by *Arabidopsis TGG1* promoter showed almost specific expression in guard cells of all tobacco organs, old cotyledon, leaf, stem, pedicel, sepal, petal, mature anther, style, and pericarp tissues. No GUS activity was found in root tissue. This is similar to what found in *Arabidopsis* (Chadchawan, 1995). However, young cotyledon and young anther wall did not show the specificity to guard cells. This may be due to the high expression in these tissues, causing the

leakage of the degraded products to other cell types. More careful experiment is needed to repeat the *gus* gene expression in these tissues at different stages.

Surprisingly the result showed the expression in pollens of transgenic tobacco. No myrosinase activity has been reported in pollens of any plants. The similarity between guard cells and pollens are the osmotic changes in the cells. In guard cell, the osmotic potential will be decrease when potassium ion induced into cell while as in mature pollen, the osmotic potential will be decrease when dehydration occurred before pollen dispersion (Taiz and Zieger, 1998). Several genes are identified to have pollen specific expression; i.e G9 gene(John and petersen ,1994) and Waxy super (+) gene (Hiruno *et al* , 1995), but none are expressed in guard cells. On the other hand, genes expressing in guard cells, *rha 1* (Terry *et al*, 1993), *AGPase* (Muller-Rober *et al*, 1994), *CdT* (Taylor *et al*, 1995), and *HBP* (Mikami *et al*,1995) have never been reported for pollen localization.

This will be another interesting research to identify the cis-element responsible for this expression pattern.

VI *Arabidopsis TGG1* promoter – *gus* gene expression in transgenic petunia.

Much lower GUS activity could be detected in petunia leaf tissues, when compared to transgenic tobacco leaf at the same age. However, the guard cell specific expression was still conserved among species, *Arabidopsis thaliana* L., *Nicotiana tabacum* L., and *Petunia hybrida* L.

VII Guard cell specific expression of *Arabidopsis TGG1* promoter in trangenic petunia.

Arabidopsis TGG1 promoter also regulated GUS expression in pollen, similar to the phenomenon occurring in transgenic tobacco. This indicated the distinct pattern of the gene expressing in plants from different family.

VIII *Arabidopsis TGG1* promoteer ─ *gus* gene expression in transgenic potatoes.

Due to the difficulty of transformation and the transplanting to soil grown condition, only the *in vitro* putative transgenic potato was analyzed for GUS activity. GUS activity in the putative transgenic potato was higher than the non- transformed tissues in the same condition.

IX Guard cell specific expression of *Arabidopsis TGG1* promoter in trangenic potatoes.

Histochemical localization could not detect any positive results. This may be caused by too low of GUS activity in these tissues and, also leaves and stems of the aseptically grown potato are small to perform the epidermal peeling method for GUS histochemical assay.

CHAPTER VI

CONCLUSION

1. *Arabidopsis TGG1* promoter can express in tobacco, petunia and potato which are the Solanaceous plant species, and contain no TGG-glucosinolate system. This implies that the expression of *TGG1* gene is independent from glucosinolate substrates.

2. Guard cell specific expression of *Arabidopsis TGG1* promoter is conserved in tobacco (*Nicotiana tabacum* L.) and petunia (*Petunia hybrida* L.) with the exception of leakage expression in pollen of these two species.

3. Age dependent *Arabidopsis TGG1* expression is also conserved in tobacco and petunia. Moreover, the *TGG1* expression level in petunia was lower than that in tobacco.

4. The remaining ambiguous point is the *Arabidopsis TGG1* cross expression in potato (*Solanum tuberosum* L. cultivar *Spunta*). Although the GUS activity could be detected in stem and leaf tissues by fluorometric assay, histochemical localization for GUS enzyme did not show any positive results. This may be due to very low level of *Arabidopsis TGG1* promoter expression and both of leaf and stem are very little, therefore the epidermal tissue could not be peeled.

Suggestion and future studies

Base on the research results, which were described previously, it should be studied further about the following approaches :

1. Screening of the T_3 progeny from the self-fertilized T_2 kanamycin resistant plants. These homozygous kanamycin resistant transgenic lines (T_3) are able to be used for further investigation.

2. Study of the enviromental factors that can be tested for potential effects on *TGG 1* promoter ─ *gus* gene regulation :
   2.1 Phytohormone treatment ; for example, auxin, cytokinin, abscisic acid, ethylene.
   2.2 Drought stress treatment.
   2.3 Wounding treatment.
   2.4 Pathogen infection.

3. The histochemical assay techniques should be studies more for some modification that may suit potato tissues.

4. Transformation of *TGG1* promoter ─ *gus* gene into other species for the confirmation of the characterization of *TGG1* promoter.

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APPENDICES

APPENDIX I

Chemical preparation

1. MS complete medium (Murashige and Skoog , 1962) per litre

Inorganic salts

NH_4NO_3	Ammonium nitrate	1650.0 mg
KNO_3	Potassium nitrate	1900.0 mg
KH_2PO_4	Potassium Phosphate dibasic anhudrous	170.0 mg
$MgSO_4 . 7 H_2O$	Magnesium sulfate	370.0 mg
$CaCl_2 . 2 H_2O$	Calcium chloride dihydrate	440.0 mg
$MnSO_4 - 4 H_2O$	Manganese sulfate	22.3 mg
$FeSO_4 . 7H_2O$	Ferrous sulfate	27.8 mg
$Na_2 . EDTA$	Disodium ethylenediaminetetraacatate	37.3 mg
$Zn_4 . 7H_2O$	Zinc sulfate	8.6 mg
H_2BO_3	Boric acid	6.2 mg
Kl	Potassium iodide	0.83 mg
$CuSO_4 . 5H_2O$	Cupric sulfate	0.0025 mg
$Na_2MoO_4 . 2H_2O$	Sodium molybdate	0.25 mg
$CoCl_2 . 6 H_2O$	Cobalt chloride	0.025 mg

Organic constituents

Surcrose	30.0 mg
Glycine	2.0 mg
Myo-Inositol	100.0 mg
Nicotininc acid	0.5 mg
Pyridoxine	0.5 mg
Thiamine . HCI	0.1 mg
Agar	8.0 gm

2. LB Medium (Luria-Bertani Medium)

: Per litre

To 950 ml of deionized H_2O, add :

- bacto-tryptone 10 g
- bacto-yeast extract 5 g
- NaCl 10 g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (~ 0.2 ml). Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle.

3. Mg/L medium

: Per litre

To 800 ml of deionized H_2O, add :

- bacto-tryptone 5.00 g
- bacto-yeast extract 2.50 g
- NaCl 5.00 g
- Na-glutamate 1.16 g
- $MgSO_4$ 0.10 g
- KH_2PO_4 0.25 g
- Mannital 5.00 g
- Biotin 1.00 mg

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 1 N NaOH . Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle.

4. Dellaporta plant extraction buffer

- 0.1 M Tris (pH 8.0)
- 0.5 M NaCl
- 50 mM EDTA
- 1.2% SDS
- 10μM mercaptoethanol

5. 0.5 m EDTA (pH 8.0)

Add 186.1 g of disodium ethylenediaminetetraacetate.2H_2O to 800 ml of H_2O . Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Dispense into aliquots and sterilize by autoclaving

6. Ethidium bromide

Add 1 g of ethidium bromide to 100 ml of H_2O (10 mg/ml)

Stir on a mangnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foi or transfer the solution to a dark bottle and store at room temperature.

7. 3 M Sodium acetate (pH 5.2 and pH 7.0)

Dosslove 408.1 g of sodium acetate.3 H_2O in 800 ml of H_2O. Adjust the pH to 5.2 with glacial acetic acid or adjust the pH to 7.0 with dilute acetic acid. Adjust the volume to 1 liter with H_2O. Dispense into aliquots and sterilize by autoclaving.

8. 3 M NaCl

Dissolve 175.32 g of NaCl in 800 ml of H_2O. Adjust the volume to liter with H_2O. Dispense into aliquots and sterlize by autoclaving.

9. 10% Sodium dodecyl sulfate

Dissolve 100 g of electrophoresis-grade SDS in 900 ml H_2O. Heat to 68^0C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 liter with H_2O. Dispense into aliquots.

10. 20 x SSC

Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml of H_2O. Adjust the pH to 7.0 with a few drops of a 10 N solution of NaOH. Adjust the volume to liter with H_2O, Dispense into aliquots. Sterilize by autoclaving.

11. 1 N NaOH

Dissolve 40 g of NaOH in 800 ml of H_2O. Adjust the volume to liter with H_2O. Dispense into aliquots and sterlize by autoclaving.

12. 1 M Tris

Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust the pH to the desired value by adding concentrated HCl.

pH	HCl
7.4	70 ml
7.5	60 ml
8.0	42 ml

13. 5 X Tris-borate

Dissolve 54.0 g of Tris base, 88.2 g of sodium citrate and 20 ml 0.5 m EDTA (pH 8.0) in 800 ml of H_2O. Adjust the volume to liter with H_2O, Dispense into aliquots. Sterilize by autoclaving.

14. 6x Gel loading buffers

- 0.25% bromophenol blue
- 0.25% xylene cyanol FF
- 15% Ficoll (Type 400; Pharmacial) in water

15. Purination solution (0.2 N HCL)

Add 40 ml of conc HCL to 960 ml of H_2O , Dispense into aliquots. Sterilize by autoclaving.

16. Denaturalization solution

Mix equal volume of 3 N NaCl and 1 N NaOH . Dispense into aliquots. Sterilize by autoclaving.

17 Neuturalization solution

Mix equal volume of 3 N NaCl and 2 M Tris, pH 7.5 . Dispense into aliquots. Sterilize by autoclaving.

18. Primary wash buffer with Urea

Dissove of 360 g Urea, 4g SDS, 25mL 20X SSC Make up to 1 L. This can be kept for up to 3 month in a refrigetor at 2-8 C. Stringency may be increased by using a lower final SSC concentration, e.g., 0.1 X SSC instead of 0.5 X SSC.

19. Secondary wash buffer

2X SSC. This can be kept for up to 3 month in a refrigerator at 2-8 ^{o}C.

20. TE buffer

10 ml of 1 M Tris-HCL pH 8.0 and 2 ml of Na2EDTA pH 8.0 are dissolved in deionized water and adjusted volume to 1 liter.

21. DNA extraction buffer of Dellaporta ' miniprep

- 0.1 M Tris pH 8.0
- 0.5 M NaCl
- 50 mM EDTA
- 10mM Mercaptoethanol

22. Chemicals for Quigen prep

Composition of buffers

Buffer	Composition	Storage
Buffer P 1 (Resuspension Buffer)	50 mM Tris.Cl, pH 8.0; 10 mM EDTA; 100 pg/ml RNase A	4 ^{0}C, after addition of Rnase A
Buffer	**Composition**	**Storage**
Buffer P2 (Lysis Buffer)	200 mM NaOH, 1% SDS	room temp.
Buffer P3 (Neutralization Buffer)	3.0 M potassium acetate, pH 5.5	room temp. or 4 ^{0}C
Buffer QBT (Equlibration Buffer)	750 mM NaCl; 50 M MOPS, pH 7.0; 15% isopropanol; 0.15% Triton X-100	room temp.
Buffer QC (Wash Buffer)	1.0 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol	room temp.

Buffer	Composition	Storage
Buffer QF (Elution Buffer)	1.25 mM NaCl; 50 mM Tris-Cl, pH 8.5; 15% isopropanol;	room temp.
Buffer QN (Elution Buffer)	1.6 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol;	room temp.
TE	10 mM Tric-Cl, pH 8.0; 1 mM EDTA	room temp.

23. Chemicals for Histochemical assay

Substrate

The substrate , 5-bromo-4-chloro-3-indolyl-B-D-glucuronic acid , or X-Glue , is the most expensive conponent of the reagent mix. We have found no differnce in the quality of X-Glue available from several suppliers. X-Glue supplied as the cyclohexylammonium salt and is stored frozen. Stock solution

Stock solution of 0.02 M X-glue

Stock solution is made up at 0.02 M , most typically in N , N' - dimethylformamide ; ddimethyl sulfoxide (DMSO) willalso work. In our hands, DMSO solutions are somewhat less stable than those made in dimethylformamide, but certainly quite adequate. Stosk solutions are aliquoted into convenient amounts (for a final concentration of 2.0 mM we find 100 ul aliquot useful) and stored in 0.5 –m; microfuge tubes at –20 C. We have stored

solution for up to 6 months with to problems. Degraded stock solution are recognized by a brow to purple color4 change. Certain plastics, such as polystyrene, will react with dimethylformamide and should not be used.

Reagent Mix of X-Glue

The reagent mix typiucally contains three components: the substrate (1-5 mM X-Glue) , the buffer (0.1 M sodium phosphate , pH 7.0) , and the oxidation catalyst (0.5 mM each potassium ferri-and ferrocyanide , pH 7.0 , plus 10 mM EDTA , pH 7.0) .

24. Chemicals for Fluorometrical assay

Solutions

Carbonate stop buffer: 0.2 M Na2CO3. For litter, mix 21.2g of Na2CO3 in a final volume of 1 litted water.

Concentrated MU calibration stock solution: uM 7-hydroxy-4-methylcoumarin (MU) in distilled water. To prepare MU standard, frist, mix 19.8 mg 7-hydroxy-4-methylcoumarin, soduim salt, into 100 ml distilled water to produce a 1 mM MU solution. Dilute 10 ul of the 1 mM MU stock. Store at 0-5 C protected from light.

MU calibration standard: 50 nM MU. To prepare the 50 nM MU calibration standard, dilute 100 ul of the 1 uM concentrated MU calibration stock solution into 1.9 ml carbonate stop buffer. This solution should be made fresh just before use.

GUS extraction buffer: 50mM NaHPO4(pH 7.0), 10 mM B-mercaptoethanol, 10 mM Na2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100. For 100 ml GUS extraction buffer:

1 M NaPO4, pH 7.0: 5 ml

2 B-Mercaptoehanol: 0.07 ml

3 0.5 M Na2EDTA, pH 8.0: 2 ml

4 30% Sarcosyl: 0.33 ml

5 10% Triton X-100: 1 ml

6 Distilled water: 91.6 ml

GUS assay buffer: 2 mM MUG in extraction buffer. To prepare 25 ml assay solution, mix 4-methylumbelliferyl B-D-glucuronide (22 mg) and extraction buffer (25 ml).

Fluorometric assay

Calibration

The DyNA Quent 200 is adjusted with the scale knob so that 50 nM MU in stop buffer equals 500 counts on display (i.e., 10 display count/nMTurn the DyNA Quent 200 on for at least 15 min before use.

1. Fill a clean glass cuvette with 1.9 ml of carbonate stop buffer (without MU). Place the cuvette into the sample chamber and close the lip. Note that the “G” mark on the top side of the corvette are in the same orientation with each reading.
2. With the scale knob set fully clockwise, bring the display to zero by adjusting the ”Zaro:” knob on the DyNA Quent 200 . This step corrects for the blank. Afternativety, note the blank value and subtract from the samlple reading.
3. Add 100 ul of the 1 uM MU standard to the carbonate blank, and mix by inversion to give the 50 nM MU standard solution. Place cuvette into sample chamber and and close lid.

4. Adjust the scale knop so the TKO 100 reads 500. Thus a 50 nM solution of MU will read 500 on the TKO 100 display; that is, 10 display counts/nM MU.

GUS assay.

1. Grind a tissue with 0.3-1 ml of extraction buffer with a mortar and pestle. Precipitate by centrifugation at 5000 RPM for 5 min in centrifuge, supernatant using for GUS enzyme solution.
2. In test tubes on ice, add:

   GUS assay buffer (2mM MUG): 10 μl

   GUS extraction buffer: 50 μl

   GUS enzyme solution: 40 μl
3. Take the test tubes containing the assay solution from ice and start the assay by placing them in a 37 ^{0}C water bath for 45 min . Immediately quench the reaction by placing into 1.90 ml stop buffer.
4. Read sample in a calibrated fluorometer.

APPENDIX II

Table11 Concentration of *Agrobacterium tumefaciens* LBA4404 containing ProA1.1.

Times	Concentration of A. tumefaciens LBA4404-ProA1.1			
	replication 1		replication 2	
	OD_{620}	concentration by visible plate count	OD_{620}	concentration by visible plate count
0	0.07	2910000000	0.09	5600000000
6	0.44	51069000000	0.47	1.02E+11
12	0.58*3	1.55838E+12	0.55*3	3.08E+12
18	0.55*5	2.26E+12	0.45*5	1E+12
24	0.59*5	3.286E+12	0.53*5	1.26E+12

Table 12 The analysis of variance (ANOVA) of GUS activity in T_2 transgenic tobacco cotyledons at different ages.

SV	DF	SS	MS	F
Treatment	4	19396.723	4849.181	4.498*
Error	15	16172.107	1078.14	
Total	19	35568.830		

CV = 21.477 %

Table 13 The analysis of variance (ANOVA) of GUS activity in leaves at different position of aseptically grown T_2 transgenic tobacco.

SV	DF	SS	MS	F
Treatment	14	11923.45	851.67	4.674**
Error	45	8200.44	182.232	
Total	59	20123.89		

CV = 49.69%

Table 14 The analysis of variance (ANOVA) of GUS activity in leaves at different position of soil grown T_2 transgenic tobacco.

SV	DF	SS	MS	F
Treatment	12	99.606	8.3	3.0701**
Error	39	87.460	2.243	
Total	51	187.065		

CV = 85.92 %

Table 15 The analysis of variance (ANOVA) of GUS activity in different tissues of soil grown transgenic petunia.

SV	DF	SS	MS	F
Treatment	6	93.86	15.64	13.205**
Error	21	24.88	1.185	
Total	27	118.74		

CV = 46.52%

BIOGRAPHY

Miss Rakchanok Koto was born on November, 10 1973 in Bangkok, Thailand. She finished her primary education from Sainumpeung school in 1991, and graduated with a Bachelor degree of Science in Biotechnology, Faculty of Science, King Mongkut Institute of Technology Ladkrabang in 1995. She has studies for Master degree in Genetic progreamme at Chulalongkron University science 1995.