TRANSGENE REPEAT FORMATION AND PROMOTER METHYLATION IN TRANSGENIC PLANTS

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(BIOTECHNOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2004

ISBN 974-04-5314-7

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was submitted to the Faculty of Graduate Studies, Mahidol University
For the degree of Master of Science (Biotechnology)
on
October 6, 2004

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ACKNOWLEDGEMENTS

I would like to express my deepest sincere gratitude to my advisor, Asst. Prof. Jarunya Narangajavana, for valuable supervisions, creative guidance and encouragement throughout the research. I deeply thank to all of my committees: Dr. Nathinee Panvisavas, Dr. Salak Phansiri and Dr. Unchera Sookmark for their useful comments and advices.

My deepest appreciation is expressed to Dr. Metinee Srivatanakul for her great advices and the experimental plan on multiple shoot regeneration system and gene transformation in shoot apical meristem of *indica* rice. Special thanks would go to Dr. Budsaraphorn Ngampanya for the system of *Agrobacterium*-mediated transformation in *japonica* rice. Without their suggestions, my research will not be completed.

Also sincere thanks to Miss Phitsamai Duangsawang for her grateful helps of *Agrobacterium*-mediated transformation in shoot apical meristem of *indica* rice and special thanks to Miss Onuma Tangsomsuk for rice tissue culture during the particle bombardment experiment. All of my friends and members of Plant Biochemistry and Molecular Genetics Laboratory, Department of Biotechnology, Faculty of Science, Mahidol University, are thanked for their wonderful friendships, great discussions and encouragement.

Thanks also indebted to the Thesis Grant, Faculty of Graduate Studies, Mahidol University and Ministry of Education, Commission of Higher Education, subproject "Graduate Study and Research in Agricultural Biotechnology" for their partial support.

Finally, I would like to express my gratitude and deepest appreciation to my beloved parents, Mrs. Kasama Raktarattana and Mr. Bumrung Yookongkaew, and my dearest brother, Mr. Araya Yookongkaew, for their infinite love, attention, understanding and encouragement, which will never be forgotten.

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TRANSGENE REPEAT FORMATION AND PROMOTER METHYLATION IN TRANSGENIC PLANTS

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ABSTRACT

Stable integration of a single copy of delivered DNA into plant cells is most desirable for production of transgenic plants. However, the majority of transgenic lines have various patterns of transgene loci. The inactivation of transgenes is often accompanied by transgene arrangement, multiple copies of the gene and promoter methylation events. This research aimed to study transgene integration patterns and promoter methylation in transgenic tobacco (*Nicotiana tabacum* cv. Samson NN), *japonica* rice (*Oryza sativa* ssp. *japonica* cv. Taichung) and *indica* rice (*Oryza sativa* ssp. *indica* cv. KDML105).

Leaf disks of tobacco were transformed via *Agrobacterium*-mediated transformation and particle bombardment. Using the former technique, the bacterial strain EHA105 harboring pCAMBIA1301, which contains β-glucuronidase (*gus*) gene and hygromycin resistant (*hpt*) gene driven by CaMV 35S promoter, was employed. All of the transgenic plants maintained GUS activities with complete T-DNA integration cassette. By contrast, 70% of transgenic lines produced by particle bombardment showed silencing of *gus* gene expression. Deletion of *gus* was detected in all silent lines.

Using *Agrobacterium*-mediated transformation, calli of *japonica* rice were transformed with EHA105 harboring pCAMBIA1301. GUS activity was detected in 43% of transgenic rice. The absence of promoter region and *gus* gene were observed in some silent lines. In *indica* rice, shoot apical meristem transformation incorporated with multiple shoot regeneration system was established in both *Agrobacterium*-mediated transformation and particle bombardment methods. The *Agrobacterium* strain EHA105 was more effective than AGL1 for gene transfer. Acetosyringone had no effect while sonication at 10 seconds significantly enhanced the transient expression. In particle bombardment, the effect of acceleration pressures, target distances and the number of bombardments were studied. None of the parameters affected multiple shoot regeneration. Southern blot analysis revealed the presence of multiple copies of *gus* gene; however, the number of transgenes had no effect on the level of *gus* expression. RT-PCR analysis showed that the levels of *gus* transcripts decreased from the intensive, moderate and silent GUS-expressing plants, respectively. Using bisulfite genomic sequencing PCR, a high level of DNA methylation in the CaMV 35S promoter of transgenic plants was observed in all silent lines. Indeed, the methylations in both symmetrical and asymmetrical sequences were crucial for transgene inactivation at the transcription level. The investigation of regulation of transgene expression in relation to transgene integration and DNA methylation may provide more information for potential manipulation of transgenic plants.

KEY WORDS: GENE SILENCING / TRANSGENE INTEGRATION / DNA METHYLATION / GENE TRANSFORMATION

189 pp. ISBN 974-04-5314-7

การจัดเรียงตัวของยีนและการเกิดดีเอ็นเอเมทิลเลชั่นบนโปรโมเตอร์ในพืชที่ผ่านกระบวนการถ่ายยีน
TRANSGENE REPEAT FORMATION AND PROMOTER METHYLATION IN TRANSGENIC PLANTS

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บทคัดย่อ

ปัญหาในการสร้างพืชดัดแปลงพันธุกรรมที่สำคัญคือการเกิด silencing หรือการไม่แสดงออกของยีน งานวิจัยนี้จึงมุ่งเน้นศึกษาอิทธิพลของการจัดเรียงตัวของยีนและการเกิดดีเอ็นเอเมทิลเลชั่นต่อการแสดงออกของยีน *gus* ที่อยู่ภายใต้การควบคุมของ CaMV 35S โปรโมเตอร์ในพืชต้นแบบคือ ยาสูบ (*Nicotiana tabacum* cv. Samson NN) ข้าว *japonica* (*Oryza sativa* ssp. *japonica* cv. Taichung) และ ข้าว *indica* (*Oryza sativa* ssp. *indica* cv. KDML105)

จากการถ่ายยีนสู่ยาสูบด้วยเทคนิค *Agrobacterium*-mediated transformation และ particle bombardment พบว่าพืชทุกต้นที่ได้จากการถ่ายยีนด้วยวิธี *Agrobacterium* มีการแสดงออกของ *gus* ในขณะที่วิธีที่สองทำให้เกิด silencing ถึง 70% อันเป็นผลมาจากการขาดหายไปของชิ้นส่วนของยีน *gus* นอกจากนี้ได้ทดลองใช้ *Agrobacterium* ถ่ายยีนเข้าสู่แคลลัสของข้าว *japonica* พบต้นพืชที่มีการแสดงออกของ *gus* จำนวน 43% และปรากฏว่าเกิดการขาดหายไปของ *gus* และ CaMV 35S โปรโมเตอร์ในข้าวบางต้นที่ไม่มีการแสดงออกของยีน ในข้าว *indica* ได้พัฒนาการถ่ายยีนเข้าสู่ shoot apical meristem ในระบบการเพาะเลี้ยงเนื้อเยื่อที่มีการชักนำให้เกิด multiple shoots เมื่อใช้เชื้อ *Agrobacterium* ในการถ่ายยีนพบว่าสายพันธุ์ EHA105 มีประสิทธิภาพมากกว่า AGL1 การใส่ acetosyringone ไม่มีผลต่อการเพิ่มความสามารถในการถ่ายยีนของเชื้อ แต่การใช้ sonication เป็นเวลา 10 วินาทีพบการแสดงออกของ *gus* เพิ่มขึ้นอย่างมีนัยสำคัญ นอกจากนี้ผู้วิจัยได้ศึกษาปัจจัยต่างๆในการถ่ายยีนด้วยวิธี particle bombardment เช่น แรงดัน ระยะทาง และจำนวนครั้งของการยิง พบว่าปัจจัยเหล่านี้ไม่ทำให้การเกิด multiple shoots เปลี่ยนแปลง จากผลการทดลองในพืชที่ไม่มีการขาดหายไปของชิ้นส่วนของยีนโดยใช้เทคนิค Southern blot analysis และ RT-PCR ปรากฏว่าจำนวน copy ไม่มีผลต่อการแสดงออกของยีน *gus* ในขณะที่ระดับการสร้าง mRNA จะลดลงจากพืชที่มีการแสดงออกของ *gus* มาก ปานกลาง และไม่แสดงออกตามลำดับ เมื่อศึกษาการเกิดเมทิลเลชั่นที่บริเวณ CaMV 35S โปรโมเตอร์ด้วยวิธี bisulfite genomic sequencing PCR พบเมทิลเลชั่นสูงทั้งในบริเวณที่เป็น symmetrical and asymmetrical sequences ของพืชที่ไม่มีการแสดงออกของยีน ซึ่งแสดงถึงการเกิด silencing ที่ระดับ transcription การศึกษาบทบาทของการจัดเรียงตัวของยีนและการเกิดดีเอ็นเอเมทิลเลชั่นต่อการแสดงออกของยีนจะนำไปสู่การพัฒนาวิธีสร้างพืชดัดแปลงพันธุกรรมที่มีประสิทธิภาพต่อไปในอนาคต

189 หน้า ISBN 974-04-5314-7

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LIST OF ABBREVIATIONS

A	Absorbance
AB medium	*Agrobacterium* culture medium
AS	acetosyringone
ATP	adenosine triphosphate
bp	base pair
BA	N^6-benzyladenine
°C	degree Celsius
Cb	carbenicillin
Cf	cefotaxime
cm	centimeter
cv.	cultivar
dsRNA	double stranded ribonucleic acid
DNA	deoxy ribonucleic acid
et al.	et. alli (Latin), and others
h	hour
hpt	hygromycin resistant gene
Fig.	figure
g	gram
µg	microgram
g	gravity
gus	β-glucuronidase gene
GUS	β-glucuronidase
Km	kanamycin
Kb	kilobase pair
LB	left border
LB	Luria-Bertani medium
M	molar
µM	micromolar

LIST OF ABBREVIATIONS (continued)

mg	milligram
min	minute
ml	milliliter
mm	millimeter
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog medium
NAA	1-naphthalene acetic acid
ng	nanogram
nt	nucleotides
OD	optical density
PCR	polymerase chain reaction
RB	right border
Rf	rifampicin
RNA	ribonucleic acid
rpm	revolution per minute
RT-PCR	reverse-transcription-polymerase chain reaction
s	second
TDZ	thidiazuron
V	volts
X-Gluc	5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid

CHAPTER 1
INTRODUCTION

Genetic engineering has opened new avenues to modify crops, and provided new solutions to solve specific needs. In the future, the proportion of acreage planted with transgenic crops, and the range of transgenic crops, is sure to increase. Beyond crop improvement, the ability to engineer transgenic plants is also a powerful and informative means for studying gene function and the regulation of physiological and developmental processes. Transgenic plants are being used as an assay system for the modification of endogenous metabolism or gene inactivation. Advances in tissue culture, combined with improvements in transformation technology, have resulted in increased transformation efficiencies in many plant species (1).

Up to the present, the most favorite techniques of gene transfer in plants are classified into 2 methods; *Agrobacterium*-mediated transformation and particle bombardment. *Agrobacterium tumefaciens* is a soil bacterium that can genetically transform a segment of DNA (transfer DNA, abbreviated as T-DNA) from a tumor-inducing plasmid (Ti plasmid) into plant cells (2, 3). This method has been routinely utilized in gene transfer of dicotyledonous plants. However, efficient transformation in monocotyledonous plants including rice has also been reported (4-7). The *Agrobacterium* system is attractive because of the ease of the protocol coupled with minimal equipment costs. Moreover, transgenic plants obtained by this method often contain simple copy insertions (1). To this end, highly efficient vectors were designed with extra copies of the virulence genes (superbinary vector), or with mutations that enhance virulence gene expression. Already, it is possible to transfer large fragments (150 kb) into plant nuclear genomes (8). Particle bombardment is the physical method that employs high-velocity metal particles to deliver biologically active DNA into plant cells which can be regenerated to whole plants. The concept has been described in detail by Sanford (9). The ability to deliver foreign DNA into regenerable cells, tissues or organs appears to provide the best method for achieving truly genotype

independent transformation bypassing *Agrobacterium* host specificity and tissue-culture-related regeneration difficulties. Due to the physical nature of the process, there is no biological limitation to the actual DNA delivery process, consequently genotype is not a limiting factor (10). However, molecular analysis of plants obtained by particle bombardment generally reveals a complex pattern of transgene integration. The transgene loci composed of multiple copies of whole, truncated, and rearranged delivered DNAs frequently organized as direct or inverted repeats that are interspersed with variable-sized genomic DNA fragments (11-13).

A tissue culture stage is required in most current transformation protocols to ultimately recover plants. Indeed, it is the totipotency of plant cells that underlies most plant transformation systems. Plants are regenerated from cell culture via two methods, somatic embryogenesis and organogenesis. Somatic embryogenesis is the generation of embryos from somatic tissues, such as embryos, microspores or leaves (1). Embryogenic tissues are, in general, very prolific and allow recovery of many transformants that are, in most cases, non-chimeric because of the assumed single cell origin of somatic embryos (14). However, the use of embryogenic tissue can have some limitations. It can be labor intensive to establish and maintain the culture and the recovery of plants can be a long process, with the risk of encountering morphological abnormalities and sterility. This system also requires a constant source of material to initiate new embryogenic cultures (1). Organogenesis is the generation of organs, usually shoots, from a variety of tissues. Except for monocot leaf explants that contain meristems only at the leaf base (14), cotyledons, leaf fragments, hypocotyls and scutella from embryos. The advantage of this system is that shoots can usually form roots readily. If roots fail to appear, grafting can be the solution (15).

The process of gene integration into plant genome is still mysterious. It has been reported that delivered DNA is integrated into plant genomes primarily through illegitimate recombination (IR). This process, which is associated with double-strand break (DSB) repair (16), is also invoked in integration of T-DNA into yeast (17) and plant genomes (18, 19). Moreover, multiple transgene cointegration has been proposed to result from homologous recombination between backbones of co-delivered plasmids, either before or after DNA integration, or from the incorporation of different transgene molecules into the same site in the genome mediated by DNA repair

processes (20). The interaction among transgene rearrangement, multiple copies or even additional copies of an endogenous gene result in homology dependent gene silencing (21, 22).

Gene silencing may occur either through repression of transcription, termed transcriptional gene silencing (TGS), or through mRNA degradation, termed post-transcriptional gene silencing (PTGS). TGS is meiotically heritable and correlates with modifications in DNA as shown by local changes in chromatin structure (23); by hyper-methylation of the promoters of silenced genes (24); or both. In contrast, PTGS is not meiotically transmitted and must be reestablished in each sexual generation. PTGS is not necessarily coupled with a modification of the DNA template, but increased amounts of DNA methylation within the protein-coding region of silenced genes have been observed (21). PTGS occurs in the cytoplasm and is associated with the appearance of specific low molecular weight RNA fragments. TGS may be established by a DNA-DNA interaction between homologous sequences (25). However, double-stranded RNA derived from promoter regions has recently been shown to be involved in the transcriptional inactivation of homologous sequences in ectopic positions (26). It is not known if DNA methylation alone is sufficient to shut off transcription. However it has been demonstrated that nucleases have reduced accessibility to a methylated silenced gene, suggesting an altered chromatin structure (27). Both TGS and PTGS lead to sequence-specific suppression of gene expression associated with methylation of the silenced loci and the formation of aberrant transcripts. It is also possible that the two are linked in these early steps of the two processes.

The experiments through this research were divided into 3 parts depending upon the plant materials for gene transfer. The first part was gene transformation in leaf disk of tobacco (*Nicotina tabacum* cv. Samson NN). The methods included *Agrobacterium*-mediated transformation as well as particle bombardment. The second part was gene transformation in *japonica* rice (*Oryza sativa* ssp. *japonica* cv. Taichung). The embryonic calli were respected to gene transfer under the technique of *Agrobacterium*-mediated transformation. The last one was gene transformation in *indica* rice (*Oryza sativa* ssp. *indica* cv. KDML105). We established the system of shoot apical meristem transformation in both *Agrobacterium*-mediated transformation

and particle bombardment. Various parameters related to transformation efficiency were evaluated and discussed.

The objectives of this research were as following:

1. To study the arrangement of transgene on transgenic plant genome
   1.1. Study the arrangement of transgene in a monocotyledonous plant (rice) and a dicotyledonous plant (tobacco).
   1.2. Compare the transgene integration pattern between *Agrobacterium*-mediated transformation and particle bombardment.
2. To study the expression of transgene in relation to the integration event of transgene
3. To study promoter methylation in relation to the level of transgene expression

CHAPTER 2
LITERATURE REVIEW

1. The transfer of foreign genes into plant genome

1.1 *Agrobacterium*-mediated transformation

Agrobacterium is a soil-born bacterium that, in the presence of a wounded plant, moves toward it, attach itself to the wound site, and proceeds to transform a large tumer-inducing (Ti) or rhizogenic (Ri) plasmid DNA into the plant cell. These genes, some of which encode enzymes for biosynthesizing plant hormones, are stably integrated into the chromosomes of a single plant cell and subsequently inherited by all the progeny of this cell. The transformed cells syhthesize excessive amounts of auxin and cytokinin, and a tumerlike growth or gall is formed (2, 3). The transferred DNA (T-DNA) is referred to as the T-region when located on the Ti or Ri plasmid. Some Ti plasmids contain one T-region, whereas others contain multiple T-regions (28, 29). T-regions are defined by T-DNA border sequences. These borders are 25 bp in length and highly homologous in sequence (30, 31). Right borders of T-DNA appears to be more important than left borders (32, 33) because the right border sequence not only serve as a target for the VirD1/VirD2 endonuclease but also serve as the covalent attachment site for VirD2 protein.

In plant genetic engineering, this mechanism of *Agrobacterium* has been served as a system to transfer an interesting gene in to plants. The genes in the Ti plasmid that cause the tumorous growth of the plant are removed and replaced with the genes someone wants to transfer to the plant. There are three phases of plant transformation:

1. Creating a strain of *Agrobacterium* that has a Ti plasmid with the interesting gene
2. Culturing the bacteria with pieces of plant tissue so that DNA transfer can occur

3. Regenerating a plant from the transformed cells at the same time as the non-transformed cells are being killed by antibiotic

Not only the success of gene transfer in plants, *Agrobacterium*-mediated transformation protocols have been adopted for non-plant as well. There are many publications describe about transformation in *Saccharomyces cerevisiae* (34, 35) and filamentous fungi (36, 37). Recently, transformation of HeLa cells mediated by *Agrobacterium* has also been described (38, 39).

1.1.1 T-DNA transferred from *Agrobacterium* to plant cells

Transformation of DNA into plant cell respects to many proteins encoded by *vir* genes in Ti plasmid. Virulence proteins have roles ranging from transcriptional activation to T-DNA processing, export, and also having a function in the host (Fig. 1). VirA and VirG proteins function as members of a two-component sensory-signal transduction genetic regulatory system. VirA is a perisplasmic antenna that senses the presence of plant phenolic compounds that are induced on wounding (40-43). In coordination with the monosaccharide transporter ChvE and in the presence of the appropriate phenolic and sugar molecules, VirA autophosphorylates and subsequently transphosphorylates the VirG protein (44, 45). VirG, on phosphorylation, it helps activate or increase the level of transcription of the *vir* genes, most probably by interaction with *vir*-box sequences that form a component of *vir* gene promoters (46, 47) and subsequence activate other *vir* gene expressions.

Within the Ti plasmid, attachments of VirD1 and VirD2 endonuclease at the T-DNA right border result in nicking of T-DNA strand (48). Cleavage of these double-stranded border sequences has been reported both *in vivo* (49-51) and *in vitro* (52). *In vitro*; however, VirD2 protein alone can cleave a single-stranded T-DNA border sequence (53, 54). Double-strand cleavage of the T-DNA border has also been noted (51, 55, 56). Following the cleavage, VirD2 remains covalently bound to the 5' end of the single-stranded (ss) T-DNA via a phosphotyrosine bond (53, 54) and protects the DNA from exonucleolytic degradation (57). It is the single stranded, and not a double-stranded T-DNA molecule, that is transferred to the plant cell (58).

Together with the VirD4 protein, the 11 VirB proteins make up a type IV secretion system necessary for transfer of the T-DNA and several other Vir proteins,

including VirE2 and VirF (59, 60). VirD4 may serve as a "linker" to promote the interaction of the processed T-DNA/VirD2 complex with the VirB-encoded secretion apparatus (61). Most VirB proteins either form the membrane channel or serve as ATPases to provide energy for channel assembly or export processes. VirB2, which is processed and cyclized, is the major pilin protein (62-64). The function of the pilus in T-DNA transfer remains unclear; it may serve as the conduit for T-DNA and Vir protein transfer, or it may merely function as a "hook" to seize the recipient plant cell and bring the bacterium and plant into close proximity to effect molecular transfer (48).

Because of its attachment to the 5′end of the T-strand, VirD2 may serve as a pilot protein to guide the T-strand to and through the type IV export apparatus. Once in the plant cell, VirD2 may function in additional steps of the transformation process. VirD2 contains nuclear localization signal (NLS) sequences that may help direct the attached T-DNA to the plant nucleus. The NLS of VirD2 can direct fused reporter proteins and *in vitro*-assembled T-complexes to the nuclei of plant, animal and yeast cells (65-69). To achieve nuclear targeting of these larger molecules, VirE2 additionally had to be associated with the T-strands. VirE2 is a non-sequence-specific single-stranded DNA binding protein (70) that can alter the DNA from a random-coil conformation to a shape that resembles a coiled telephone cord (71). This elongated shape may help direct the T-strand through the nuclear pore. Furthermore, VirE2 also contains NLS sequences that can direct fused reporter proteins to plant nuclei (65, 69). Finally, VirE2 may protect T-strands from nucleolytic degradation that can occur both in the plant cytoplasm and perhaps in the nucleus (72).

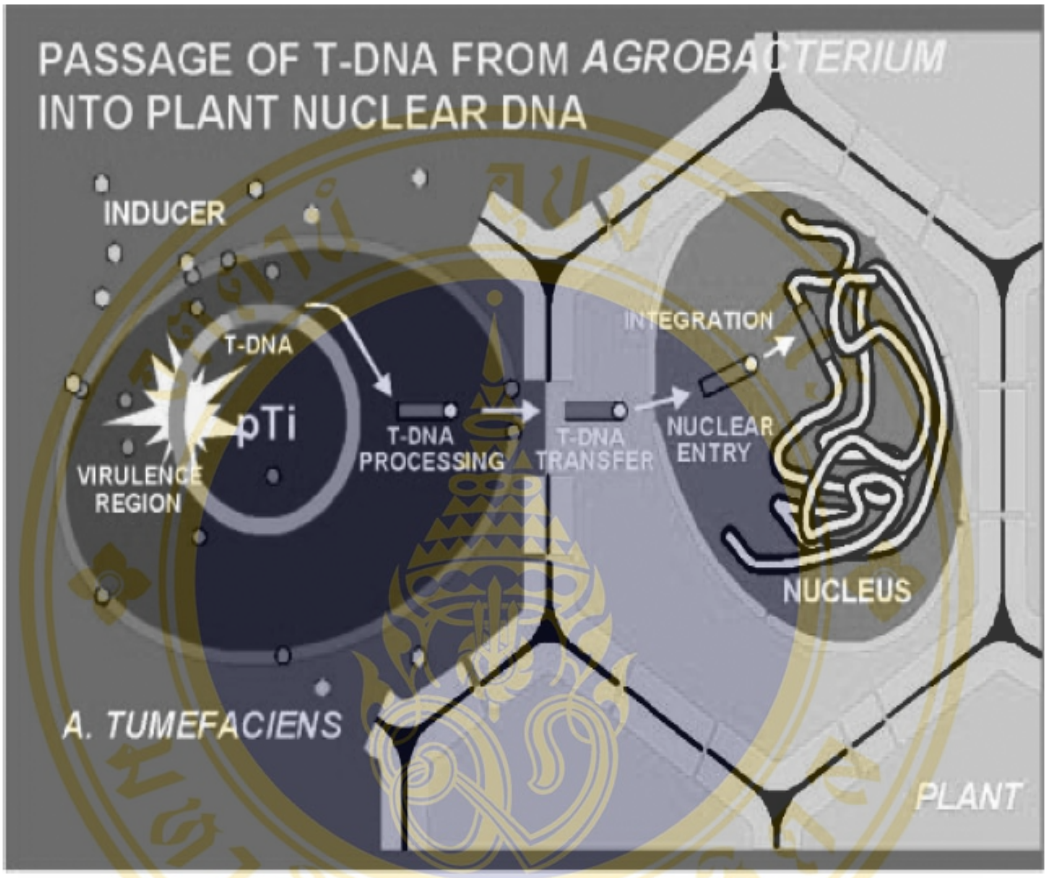

Figure 1. The path that the T-DNA travels from the stage of T-DNA processing from the Ti plasmid to stable integration into the plant genome (73).

1.1.2 T-DNA integration into plant genome

Most recently, Alonso *et al.* (74) revealed that, from analysis of 88,000 T-DNA insertions of the SALK library, integration of fewer T-DNA was found at the centromeric region. The observation of the random, as opposed to targeted, nature of T-DNA integration has led to the suggestion that the likely mechanism is through nonhomologous end joining (NHEJ). NHEJ is the DNA repair mechanism that joins double-stranded breaks (DSBs) irrespective of sequence (73). This is the process involved in well-characterized cases such as variable diversity-junction (VDJ) joining in the mammalian immune system (75). NHEJ is an unfaithful repair mechanism that often incorporates filler DNA, such as T-DNA, into the repair site.

The current model of T-DNA integration has been reported from Brunaud *et al.*(76) (Fig. 2). The authors concluded that there are microsimilarities involved in the integration of both the right and left borders. These similarities need only over a stretch of 3 to 5 bp and can be between any T-DNA and genomic sequence. This basically allows T-DNA to integrate at any locus in the genome. It was also reported that AT-rich region was often found upstream of the insertion site, which would account for the high number of T-DNA found in promoter regions and may be an indication of integration mechanism. In addition, using VirD2 as bait in a yeast two-hybrid assay, allows new insights into the alternative integration mechanism. VirD2 was shown to interact with *Arabidopsis* TATA-binding protein and CAK2M, a nuclear kinase (77). The CAK2M kinase also interacts with RNA polymerase II (C-terminal domain). These reactions together are suggestive of a role for transcription and transcription-coupled repair in T-DNA integration.

A growing concern is raised over the integration of DNA fragments outside T-DNA in the vector, which is often referred to as “backbone sequence” (78). Such integration has been found in 33% (79), 33% and 62% (80) and between 20% and 50% (81) of transformants. It is believed that the backbone sequences were integrated as a result of “read-through” at the left border in many of the cases. This means the T-strand formation initiated at the right border did not terminate at the left border. Fragments outside the right border could be integrated into plants when the entire vector plasmids were copied to the T-strand. An attempt was made to insert a lethal gene into the non-T-DNA portion of a binary vector to reduce beyond border transfer

(82). Integration of the backbone sequence was greatly reduced with the vector without much decrease in the transformation efficiency.

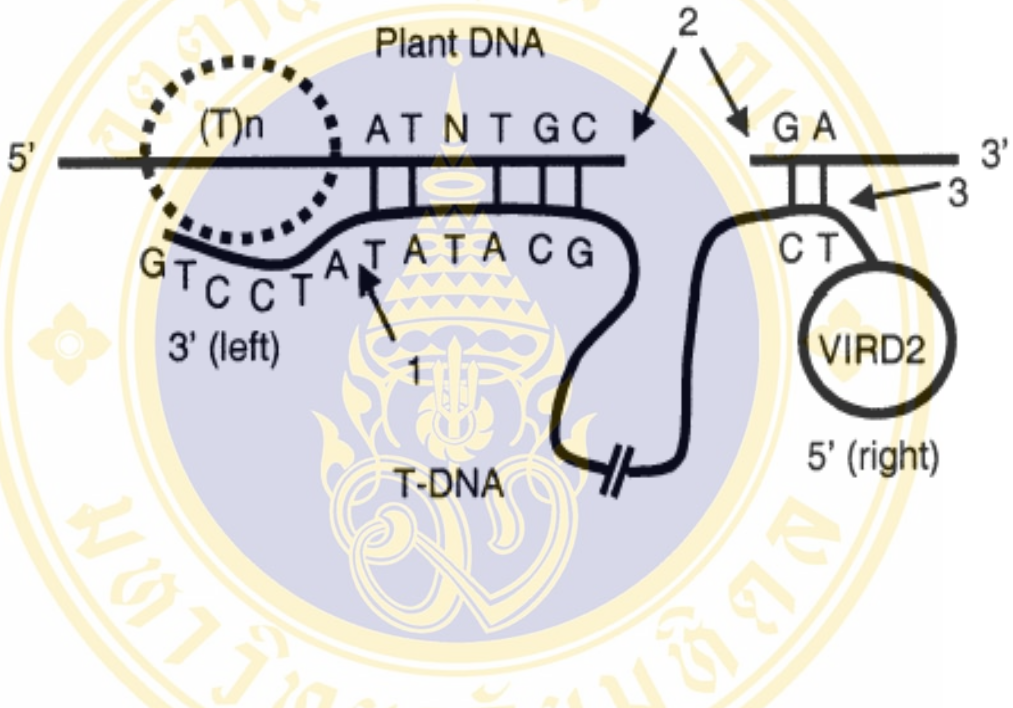

Figure 2. A model of the T-DNA integration process

AT-rich region [T(n)] operates as a preferential site of entry of the T-DNA left border. The T-DNA scans the plant DNA until it finds a microcomplementarity just downstream of the AT-rich region. A nick (2) is generated in the host DNA downstream of the microcomplementarity-based duplex and used as a priming site to synthesize the complementary strand of the T-DNA until the right border covalently linked to VirD2 is reached (76).

1.1.3 Copy number and rearrangement of DNA

Integration into plant genomes of few copies of transgenes without much rearrangement is often stated as an advantage of gene transfer mediated by *Agrobacterium.* However, full sets of information of number of copies, number of loci and integrity of T-DNA in a large number of transformants have not often been documented. In typical cases (5, 83), 20-30% of transformants carried only a single copy of the T-DNA, two or more copies of T-DNA were integrated to a single locus in more than half of the transformants, and DNA fragments that were not expected from the structure of the vector were found in less than 5% of the transformants. Multiple copies of T-DNA were integrated at a single locus in between 21% and 44% of transformants in some reports (84-86). Some of the T-DNA copies were precisely joined together, while others were separated by between a few bases and 300 bases of filter DNA, which were either derived from the T-DNA or host plants. It was proposed that these were probably the results of the multiple, independent integration of the T-DNA to receptive site (84-88). Multiple copies of T-DNA could also be inserted separately at different loci in a plant but this kind of integration is less than 10% (5, 83).

Extensive southern hybridization occasionally detected DNA fragments that were not predicted from the map of the T-DNA and/or plant DNA. Fortunately, transgenic plants showing such hybridization patterns have not frequently been found, less than 5% (5, 83) thus not rising serious concerns. Nevertheless, analysis of transformants by southern hybridization with various probe segments and restriction enzymes, and sequencing of junctions between T-DNA and plant DNA would be very important.

1.2 Particle bombardment

Particle bombardment is a direct gene transfer method that employs high-velocity metal particles to deliver active DNA into plant cells, which can be regenerated to whole plants. The ability to deliver foreign DNA into regenerable cells, tissues or organs appears to provide the best method for achieving truly genotype independent transformation by passing *Agrobacterium* host specificity and tissue-culture-related

regeneration difficulties. Due to the physical nature of the process, there is no biological limitation to the actual DNA delivery process; moreover, genotype is not a limiting factor (10).

The earliest use of microprojectiles to facilitate gene transfer into plants was reported by MacKenzie *et al.*, 1966 (89). The authors described the use of an "air blast inoculator" to spray carborundum particles onto leaves in order to wound the plant cells and promote the uptake of infectious nucleic acids and viral particles. Since the viral nucleic acids replicated soon after entering the plant cell, the efficiency of gene transfer in this procedure was not an important issue. However, this type of inoculation method is not efficient when non-infectious nucleic acids are to be introduced into plants. Particle bombardment as we know it today was developed by Sanford *et al.*, 1987 (9). The investigator described a modified 0.22-caliber shot gun in which a plastic bullet covered with DNA-coated tungsten particles (1-4 μm) was placed over the gunpowder cartridge. When the gun was fried, the plastic bullet was propelled into a solid obstruction, but this "stopping plate" contained a small aperture through which the microprojectiles continue towards their target. In this manner, the small metal particles were accelerated into plant cells, reaching an impact velocity of around 250 m/sec, which is sufficient to penetrate the cell wall. In the initial experiment, intact onion epidermis was bombarded with tungsten particles coated in tobacco mosaic virus (TMV) RNA. Onion epidermis was used because it provided a monolayer of large cells, allowing penetration and cell viability to be easily verified. Three days after bombardment, approximately 40% of the onion cells that contained metal particles showed evident of TMV infection (9).

1.2.1 Physical and chemical principles of particle bombardment

Particle bombardment is a non-biological DNA transfer process thus only physical and chemical principles are important in the success of an experiment. These principles can be divided into two groups: those concerning the properties of the microprojectile/DNA complex and those concerning microprojectile delivery and its effects on the target tissue. These principles are shown in table 1.

To perform a bombardment, tungsten and gold have been shown to meet the most appropriate particles. Tungsten is more reactive than gold and can cause

oxidation damage resulting in greater target cell toxicity. Thus, for certain sensitive tissues, the use of gold particles results in higher transformation efficiencies. However, since gold is an inert metal, it binds DNA less efficiency than tungsten (90). Recent studies have suggested that tungsten particles may catalyze the hydrolysis of phosphodiester bonds, resulting in the induction of nicks and breaks in covalently closed plasmid DNA (91) while similar studies have not been reported for gold particles.

Conformation of the transforming DNA has also been mention. Different forms of DNA (circular or linear, double stranded or single stranded) could influence transformation efficiency. Several investigators have suggested that linear DNA is a better substrate for integration than circular DNA (92) but others have found that this make no difference (93). All four forms of DNA (double stranded circular, double stranded linear, single stranded circular, single stranded linear) have been compared in parallel experiments and have been shown to generate transformants with similar transformation patterns (94). One property of the transforming DNA, which is likely to have a significant effect on transformation efficiency, is it overall length, since larger molecules may be more likely to shear either during particle acceleration (90). It has also been suggested that treatment during particle coating can improve transformation efficiency by causing the denaturation of plasmid DNA (95).

Table 1. Physical and chemical parameters affecting the efficiency of particle bombardment (90)

Parameters	Comments
Properties of the microprojectile/DNA complex	
Particle composition	Gold or tungsten
Particle size	Usually between 0.6 and 1.2 μm
Uniformity of size	Gold generally more uniform the tungsten
Particle shape	Near spherical to prevent agglomeration
Conformation of DNA	Circular or linear, single or double stranded
Loading rate	Generally 2-5 μg DNA per bombardment
Shot load	Up to 350 μg particles per bombardment
Loading efficiency	Can be highly variable
Particle delivery	
Velocity of particle impact	Depend on instrument settings: adjust force of acceleration, distance of carrier travel, distance to target, hardness of vacuum
Particle scatter	Depend on instrument design
Tissue damage	Depend on instrument design: may reflect impact of microprojectiles, carrier shrapnel, acoustic shock, or gas venting

1.2.2 Transgene integration event

Transgene integration in particle bombardment is more complicate than *Agrobacterium*-mediated transformation. This is because DNA introduced by bombardment process lacks any particular sequence motifs and is not complexed with bacterial proteins. It is likely that actual sequence has little to do with the propensity of foreign DNA to integrate since multiple-plasmid co-transformations are unbiased. Chen LL *et al.,* 1998 (96) noted that there was no preference for the integration of particular transgenes in rice plants co-transformed with up to 13 plansmids, indicating that the insertion mechanisms operated independently of gene sequence.

The advantage of *Agrobacterium*-mediated transformation is its tendency, in most plant species, to produce low-copy-number transgenic loci with simple organization and few rearrangements. Particle bombardment, in contrast, often generates much larger transgenic loci, containing from 1-20 (or more) copies. Locus structure varies considerably, encompassing single copies, tandem or invert repeats, concatemers, intact transgenes, truncated and rearranged sequences and interspersed genomic DNA (90). The complexities of transgene integration can be observed through southern hybridization and, recently, fluorescence *in situ* hybridization (FISH). The latter have generated the new models of transgene integration mechanisms in particle bombardment. The study of transgene organization in wheat by Jackson *et al*., 2001 (97) revealed that, from fibre-FISH data, transgene loci in bombarded wheat could be organized in three ways. The simplest arrangement, described as type III locus, is an intact, single-copy integration event. Type III loci may be present uniquely in a plant, or there may be two or more unlinked inserts representing multiple genetic loci. These two possibilities can be distinguished by FISH to metaphase chromosomes and genetic segregation analysis.

Other loci, described as type I loci, are longer than the single plasmid copy. Jackson *et al.* (97) reported type I transgenic loci with a continuous signal of 77 kb, representing 11 contiguous plasmid copies. These loci are concatemers of the transforming plasmid and are characterized by the absence of intervening genomic DNA. In earlier reports, the presence of concatemers was comfirmed by southern hybridization and sequencing across plasmid/plasmid junctions. The loci have been described by Kohli and colleagues as “transgene array” (98, 99).

The remaining fibre-FISH patterns, termed type II loci (97), are the most complex. They are characterized by fluorescent signals that extend for a significant distance (>100 kb) over the chromosome, but which are punctuated regularly by intervening segments of genomic DNA. Such loci have been identified in transgenic oat, rice and maize (11, 98, 100). Kohli and colleagues have named such loci "transgene clusters". Svitashev and Somers (12) showed, in one transgenic oat line, that type II loci contain genomic interspersions ranging from a few tens of base pairs to approximately 10 kb.

1.2.3 Model of transgene integration mechanism

In summary, the analysis of plasmid/plasmid and plasmid/genomic junctions in transgenic plants generated by particle bombardment reveals several features characteristic of illegitimate recombination as described (90).

- Regions of microhomology at the recombination junctions, that is, 4-8 nucleotides in common between the recombination partners.
- The presence of filler DNA at the junctions, that is, several nucleotides that are not recognizable as belonging to either recombining partner.
- Deletions of a few nucleotides in one or the other of the partners, probably reflecting nuclease "nibbling" of the foreign DNA prior to joining.
- Purine-rich elements surrounding the junction site, with similarity to topoisomerase I binding/cleavage sites.

As mention above, the integration pattern in transgenic plant could be divided into three ways: contiguous arrays (type II), interspersed clusters (type III) and widely dispersed FISH signal (type I). Any model for transgene integration following particle bombardment has tried to account for these three-way organizations. Two-phase transgene integration mechanisms have been proposed by Kohli *et al.*,1998 (98) to explain the first two levels of organization. In the "preintegration" phase, transforming plasmid molecules (either intact or patial) are spliced together. Penetration of the cell is proposed to elicit a wound response, which would include the induction of DNA repair enzymes such as nucleases and ligases. The presence of these enzymes and an excess of foreign DNA would result in the linking together of several copies to form concatemers, which would be the substrates for integration. Thus, this give rise to

rearranged transgenic sequences that do not contain any interspersed plant genomic sequences. For the second phase, the "integration" event, occurs when the integration of transgenic DNA into plant genome is initiated by illegitimate recombination at the chromosome break. The original site of integration acts as a "hot spot", facilitating subsequent integration of successive transgenic molecules at the same locus. This transgenic locus may contain plant DNA separating the transgenic sequences resulted in the interspersed clusters. Indeed, this might be due to the presence of local repair complexes that slide along the DNA and introduce nicks which can be exploited by more foreign DNA (20). Pawloski and Somers (1998) suggested an alternative second phase where a number of discrease transgene concatemers integrate simultaneously at a site containing multiple replication forks. Although there is no direct evidence for either mechanism, it is interesting to note that DNA integration is stimulated in rapidly dividing cells, and is blocked in *Arabidopsis* mutants lacking essential components of the DNA recombination machinery (101).

Moreover, from the observation of metaphase FISH chromosome, the effect of transformation event in transgene integration region has been reported. It is possible that the metal particle caused damage to a particular area of the chromatin, which is arranged in loops attached to the nuclear matrix. Each of these sites could act as a nucleation point where foreign DNA diffusing from the metal particle is used to patch up double strand breaks, generating widely separated arrays and/or clusters (12).

2. Shoot apices: materials for gene transfer

2.1 Development of shoot apical meristem

There is very fundamental difference in the way plants and animals develop. Plants have small regions, called meristems that are permanently embryogenic; that is, they can continuously give rise to new organs. This gives plants a rather simple way of solving the problem of aging: when an organ is getting old, it is allowed to die and a new one is made. Leaf shedding is a typical example of this phenomenon. When leaves are young, they photosynthesize vigorously, but as they get older their rate of metabolism starts to drop. Eventually they age and are allowed to die. In perennial

plants, new leaves are made in each growing season, whereas in annuals new leaves continue to be made at the top of the plant while older leaves near the bottom are dying.

At the top of each shoot is an apical meristem where the cells are continuously dividing (Fig. 3). Small protrusions of dividing cells, called primordia, arise on this dome-shaped mass of dividing cells, and one by one these primordia grow out into small embryonic leaves or become dormant lateral buds. Together, the apical meristem, the primordia, and the tiny leaves make up the apical bud that is found at the end of every twig. Apical buds are also present at the ends of other shoots, but are not always as easily identified as in twigs. The activity of meristems is regulated by the environment (temperature and day length) as well as by hormones. When apical buds are actively growing, they produce the growth hormone auxin, and auxin regulates the elongation of the cells just below the meristem.

The function of the shoot apical meristem is to produce the cells that make up the shoot system, leaves and stems. However, given the right environmental and/or hormonal stimuli the activity of this meristem can be redirected toward making flower primordia instead of leaf primordia. These flower primordia then form sepals, petals, stamens, and anthers. At that point, the activity of the meristem ceased, and whatever cells remain, stops dividing. That is the normal process in many annual plants in which the apical bud first gives rise to a number of leaves and finally forms the flower (102).

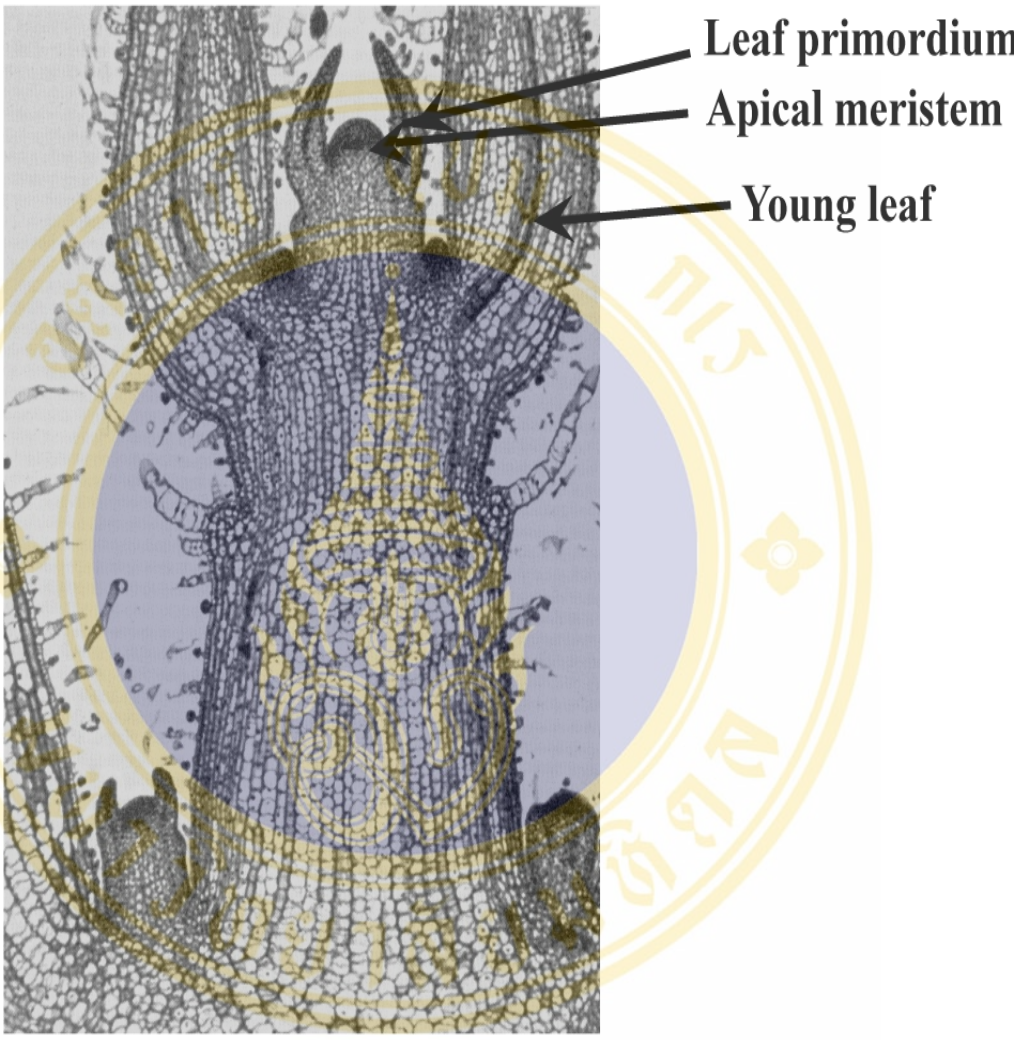

Figure 3. Longitudinal section through the shoot apex showing the location of the meristem and two leaf primordia (102)

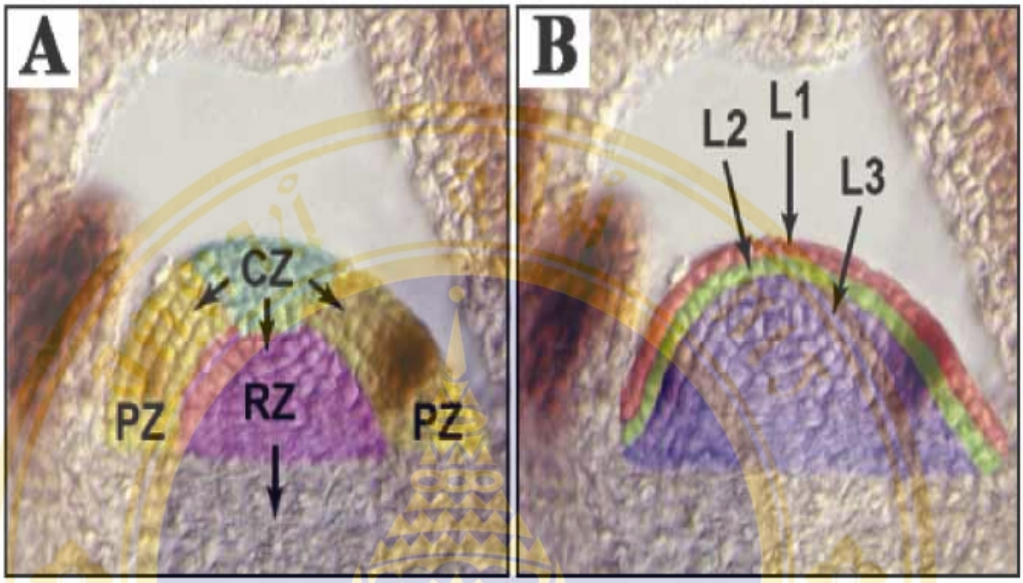

Figure 4. Histology of the shoot apical meristem (SAM)

(A) Lateral organs are produced from cells recruited from the peripheral zone (PZ), whereas the bulk of the stem is derived from cells recruited from the rib zone (RZ; the outermost layers of the stem are derived from the peripheral zone). The central zone (CZ) acts as reservoir of stem cells, which replenishes both the peripheral and rib zones as well as maintaining the integrity of the central zone itself. (B) The SAM is composed of clonally distinct layers of cells. In the SAMs of eudicot plants, there are typically three layers. However, the SAMs of many monocots, including grasses, are composed of only two layers. The epidermal layer (L1) forms one clone, its integrity being maintained by the almost exclusively anticlinal orientations of cell division within the layer. The subepidermal layer (L2) also exhibits almost exclusive anticlinal orientations of cell division, which maintain its clonal distinctness. The L1 and L2 are collectively referred to as the tunica. Cells interior to the L2 constitute the corpus (L3), in which various planes of cell division are observed (103).

2.2 Gene transformation in shoot apices

The two most critical steps which much be mastered for the transformation of plants are the transfer of the foreign DNA into plant cell and the regeneration of plants from the transformed cells. In many species, the recovery of transgenic plants is difficult or impossible because cells that are accessible to gene transfer may not be competent for plant regeneration. Thus, the development of simple and efficient approach for gene transformation is of major interest. Shoot meristem and apex cultures became popular in the ornamental nursery industry after the discovery that rapidly growing shoots of many virus infected clones could be free of virus and used to produce virus-free germplasm (104). Over time, it was observed that the incidence of genetic mutations and somaclonal variation was low in plants regenerated from shoots. One of the reasons for this low mutation frequency may be the absence of tissue dedifferentiation steps that are common in the initiation of callus and somatic embryo cultures. This event is known to trigger retrotransposon activity in cultured plant tissues and produces permanent mutations (105).

Smith *et al.*,1988 (106) and McCabe *et al.*,1988 (107) have first proposed an approach based on the transformation of shoot apices. Culture of shoot apices can be combined with either *Agrobacterium*-mediated transformation or particle bombardment (108-111). It has been proposed that the efficiency of *Agrobacterium*-mediated transformation in shoot apices of sunflower (*Helianthus annuus* L.) could be increased by sonication and the assessment of macerating enzymes such as cellulase and pectinase (105, 112). Moreover, shoot meristematic cultures (SMCs) induced from shoot apices of germinating mature seeds of oat plants (*Avena sativa* L.) have been shown as a good material for particle bombardment with high transformation frequency compared with callus tissues and leaf base derived cultures (110).

There are two possibilities for recovering transgenic plants via transfer DNA into the shoot apical meristem. One possibility is that transgene progeny may be directly produced from the meristem cells followed by the development of a partially transgenic reproductive organ. In this case, the primary transformants will always be

chimeric. An alternative possibility is to multiply transgenic apical meristem cells, which can be reprogrammed into developmental direction under *in vitro* condition (113). Thus, manipulation of the transgenic meristem cells by growth regulator treatments that induce multiple shoot regeneration from the shoot meristem could generate more stable transformants. Thidiazuron (TDZ), a phenylurea-type cytokinin, is the one that has been reported to facilitate multiple shoot proliferation in many plants (111, 114, 115). Addition of 1 μM TDZ in multiple shoot induction medium resulted in high number of regenerated shoots per explant in kenaf-shoot apex culture (115).

3. DNA methylation

DNA methylation is an important modification of DNA that involves in genome management and in regulating gene expression during development. Methylation is carried out by DNA methyltransferases which catalyse the transfer of a methyl group to bases within the DNA helix (Fig. 5). DNA methylation plays an important role in recognition and protection of self DNA as well as DNA repair and replication (116). For mammalian development, the enzymatic methylation of cytosine within the context of a simple dinucleotide site, CG, is essential in controlling several biological process such as X chromosome inactivation, genomic imprinting, genomic stability and chromatin structure (117). In plants, methylation is essential for defense mechanism that against invading DNA and transposable elements (118, 119). Moreover, cytosine methylation involves in the regulation of developmental processes that take place in specific tissues or stages of development (120, 121). Besides, demethylation of the promoter region promotes flowering in plants from the vernalization-responsive ecotype (122).

There are 3 classes of cytosine methyltransferase in plants based on their enzyme structure and similarity of conserved amino acid motifs (123). The first class is the DNA cytosine methyltransferase MET1 family, homologues of mammalian DNMT1 (124-126). MET1, the predominant family member is responsible for the maintenance of DNA methylation at CpG sites (127-130). Depletion of MET1 DNA methyltransferase in *met1* mutants and *asMET1* results in the loss of the majority of

CpG methylation at the hypermethylated centromeric repeats, transposon-related sequence, and the silent transgenic loci (127, 129). *met1* mutants also show numerous development phenotypes, which become more severe with each successive generation and include alterations in flowering time, homeotic floral mutations and reduced fertility (127, 128). Moreover, MET1 was shown to be important for the maintenance of CpG methylation, and thus of $H3K9^{met}$ in *met1-3* was detected in the heterochromatic loci tested (131) representing the relationship between DNA methylation and histone methylation in maintenance of gene silencing.

A second class of methyltransferase was identified by Henikoff and Comai (132) when searching the plant database for proteins containing chromodomain motifs (133). A small gene family encoding chromomethylases (CMT family) with at least 3 members has been identified in *Arabidopsis* (134, 135). They are characterized by the presence of a chromodomain located between conserved motifs (135). Amino acid sequence analysis using RPS-BLAST program available in NCBI database revealed the presence of one BAH domain in amino-terminal domain of chromomethylases. These studies of *zmet2* and *cmt3* mutants showed that these two enzymes are responsible for maintenance methylation activity at CpNpG content in plants (136, 137). DNA methylation in *cmt3* mutant also revealed silencing in retrotransposons (137, 138).

The other class of DNA methyltransferase in plants is DRM2 (Domain Rearranged Methylase2), which was isolated from *Arabidopsis.* The similar enzyme, ZMET3, was also discovered in maize (139). Both DRM2 and ZMET3 contain a motif with ubiquitin-associated (UBA) domains at the N-termini (139). DRM2 class has been demonstrated to be required for *de novo* CpG methylation at a late flowering gene as well as asymmetric methylation at SUPERMAN locus in *Arabidopsis* (140).

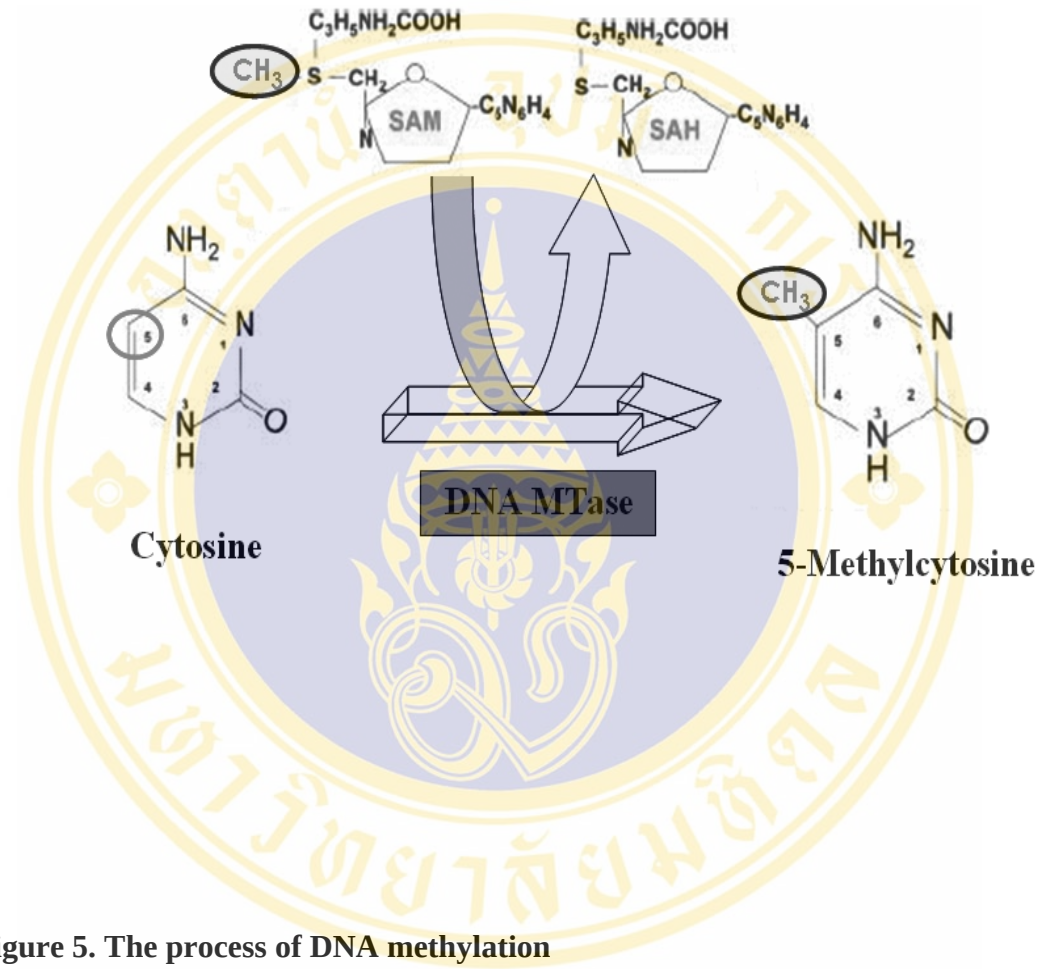

Figure 5. The process of DNA methylation

A methyl group is transferred from s-adenosyl-L-methyonine (SAM) to carbon position 6 of cytosine residues by DNA methyltransferase (DNA MTases). This figure was modified from Attwood *et. al.*, 2002 (141).

4. Silencing of transgene in plants

Predictable, stable transgene expression is often a critical parameter for the broad use of transgenic plants. Unfortunately, transgene expression often varies over several orders of magnitude, and gene silencing is frequently observed. The first evident of gene silencing was first discovered in 1990 by Napoli *et al.* (142). They tried to overexpress the chalcone synthase (CHS), the enzyme involved in anthocyanin pathway, in pigmented petunia petals. Unexpectedly, anthocyanin biosynthesis was blocked by co-inhibiting endogenous and introduced CHS gene expression; hence, this phenomena was termed co-suppression (142). The similar evidences have been reported in *Neurospora crassa* as quelling (143) and RNA interferance (RNAi) in *Caenorhabditis elegans* (144).

Silencing in plant, not only serves as an essential component of the defense system being targeted against transposable elements and viral infection, but also plays important roles in the regulation of endogenous gene expression (145). As demonstranted by the spread of systemic silencing after localized virus infection and grafting experiments, the signals of intracellular silencing and be transmitted systematically from cell to cell over a long distance through the phloem (146, 147). Gene silencing can occur either through repression of transcription, termed transcriptional gene silencing (TGS), or through mRNA degradation, termed post-transcriptional gene silencing (PTGS).

4.1 Transcriptional gene silencing (TGS)

In plants, transgenes insert into the genome apparently at random by illegitimate recombination as described briefly above. Therefore the number of inserted copies, their chromosomal location and their local arrangement (tandem insertion, rearrangements, etc.) vary between one transformant and another (148). An inverse correlation between copy number and the level of gene expression has been reported, which suggests that increasing the number of copies of a particular gene can lead to gene silencing (148, 149). This is, because pairing between closely linked of repeat

copies results in the formation of secondary DNA structures that is difficult to transcribe, and possibly akin to heterochromatin (150).

During the last several years, the idea of short RNA can target gene silencing in both TGS and PTGS has gained tremendous momentum. The first example of an RNA-guided epigenetic modification was discovered in 1994 by Wassenegger *et al.* (151) in viroid infected plants named RNA-directed DNA methylation (RdDM). During viroid replication, cDNA copies of the viroid that had been integrated into the plant genome became methylated *de novo*. This suggested that the replicating viroid was somehow initiating methylation of the homologous DNA copies. RdDM has subsequently been shown to require a dsRNA that is processed by a dicer activity into short RNAs 21-26 nt in length (152, 153), moreover, a longer class of short RNAs 24-26 nt in length has also been identified (154, 155). dsRNAs that contain sequences identical to promoter regions can induce methylation of unlinked homologous promoters resulted in transcriptional gene silencing (TGS) (26, 152, 153, 156, 157). The model of dsRNA-directed TGS was shown in Fig. 6. Moreover, the short RNAs, in conjugation with one or more DNA methyltransferases (DMTases) and possibly chromatin-modifying factors, are thought to trigger *de novo* methylation and TGS of the homologous promoter at the target locus (156). The enzymes that involve in *de novo* methylation are DMTases include Dnmt3a and Dnmt3b in mammals (158, 159) and their plant homologs, the domains rearranged methyltransferases, DRM1 and DRM2 (140). As mention earlier, methylation at symmetrical CpG and CpNpG by either MET1 or CMT family are shown to maintain methylation in newly synthesized DNA strand so that if methylation is observed in asymmetrical C, this can be taken as a measure of ongoing *de novo* methylation (156, 160).

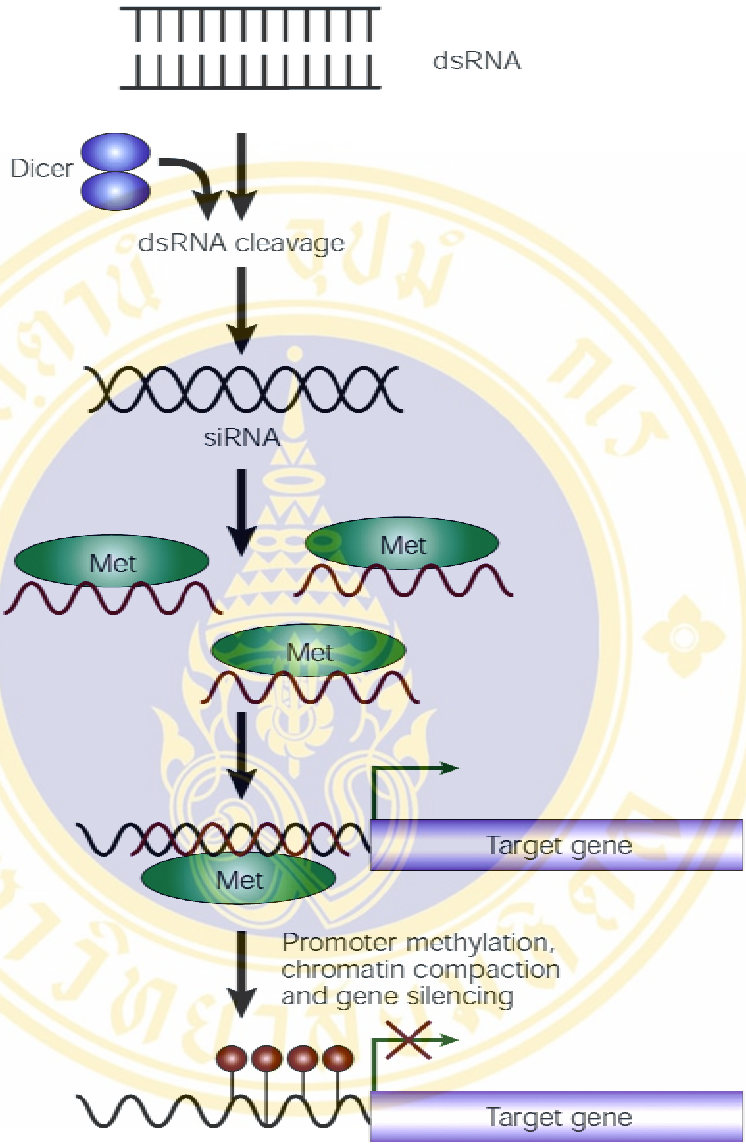

Figure 6. The model of dsRNA-directed transcriptional gene silencing in plants dsRNA directed against promoter sequences gives rise to the methylation of cytosine residues at the targeted DNA sequence. This methylation directly, or indirectly, causes changes in the conformation of local chromatin, resulting in gene silencing by loss of transcription. dsRNA, double-stranded RNA; Met, *de novo* methyltransferase; siRNA, small interfering RNA. This figure was modified from Waterhouse, PM and Helliwell, CA. (161).

Alternatively, additional mechanisms for gene suppression that related to histone deacetylation has been reported elsewhere (162-164). Several methylcytosine-binding-proteins (MBPs), such as MeCP1, MeCP2, MBD1, MBD2, MBD3 and MBD4 that interact with DMTase could recruit the repressive complexes and histone deacetylases. MBPs bond to CpGs in the promoter region form complexes with histone deacetylases and corepressors, leading to histone deacetylation, chromatin condensation and a transcriptionally inactive chromatin structure (165).

4.2 Post transcriptional gene silencing (PTGS)

Post transcriptional gene silencing is a sequence specific degradation process of RNA derived from endogenous and/or transgenes in plants transformed with homologous sequences of this gene. In a broad range of eukaryotic organisms, PTGS is triggered by dsRNA, which may be naturally derived from the transcription of inverted-repeat loci or replicating exogenous RNAs by host- or viral-encoded RNA dependent RNA polymerase (RDRP) (166-168). Viruses and transgenes are two main exogenous factors inducing the formation of dsRNAs in plants during PTGS. The viral replication by a virus-encoded RDRP in most single-stranded RNA viruses generates a dsRNA intermediate, which contains the sequence-specific information to guide a plant to protect itself by degrading viral RNAs (168). Comparatively, the generation of dsRNA initiated by transgenes may have distinct mechanisms according to the transgene structure and its integration status in the plant genome. PTGS caused by the transgene overexpressing an endogenous mRNA requires a putative plant-encoded RDRP, SGS2/SDE1 (Arabidopsis) for example, which synthesizes dsRNA intermediates from the template of aberrantly expressed single-stranded RNA (166, 167, 169). However, the requirement for RDRP is bypassed if a transgene is designed to create a dsRNA structure, or multiple copies of a transgene are integrated as inverted-repeat insertions in the genome, in which dsRNA is potentially generated by read-through transcription (170-172).

The mechanism of RNA silencing induced by dsRNA can be simplistically summarized in Fig. 7 as having two major steps; initiation and effecter steps (173). The initiation step involves the cleavage of the triggering dsRNA into siRNAs of 21-

26 nt with 2-nt 30 overhangs, which correspond to both sense and antisense strands of a target gene (145, 174). In the effecter step, the siRNAs are recruited into a multiprotein complex referred to as the RNA-induced silencing complex (RISC), in which the degradation of target mRNAs occurs with the siRNA as a guide (175, 176). Each RISC appears to have a single siRNA, an RNase (and may even have two separate RNase exonucleolytic and endonucleolytic), and an mRNA homology recognition and binding domain. Further, there are two size classes of siRNA in plants (169, 177, 178), namely, 'long siRNAs' of 24-26 nucleotides that are involved in triggering systemic gene silencing, and the 'short siRNA' (21-22 nucleotides) correlated with sequence-specific degradation of target mRNAs. Such cleavage of the target mRNA occurs almost at the middle of the siRNA within the relevant RISC (guide RNA), resulting in over 90% inhibition of target gene expression.

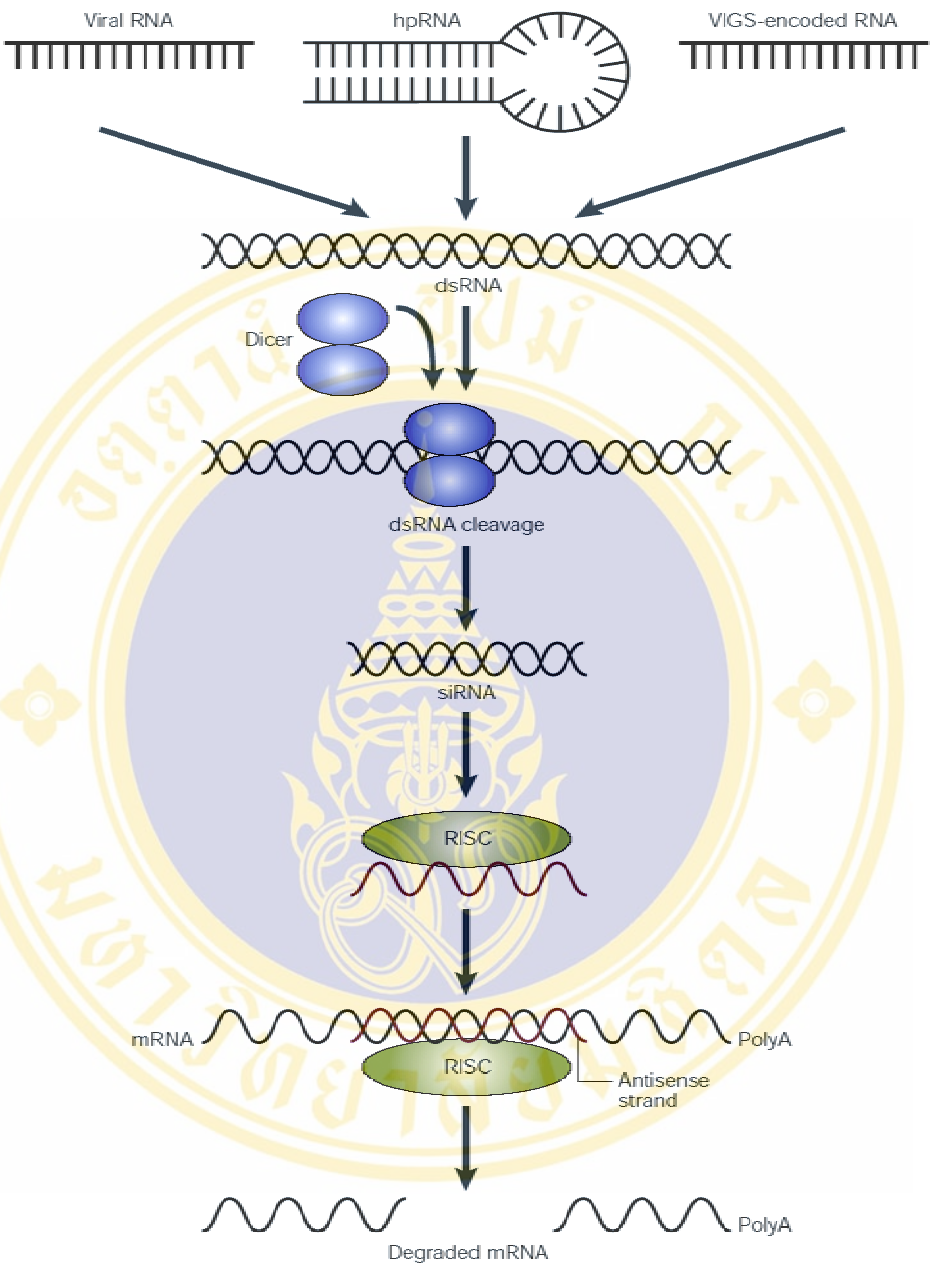

Figure 7. The model of RNA-mediated PTGS in plants

Double-stranded RNA (dsRNA) from replicating viral RNA, viral-vector-derived (VIGS, or virus-induced gene silencing) RNA or hairpin RNA (hpRNA) transcribed from a transgene, is processed by a Dicer-containing complex to generate siRNAs. An endonuclease-containing complex (called the RNAi silencing complex, RISC), is guided by the antisense strand of the siRNA to cleave specific mRNAs, so promoting their degradation (161).

CHAPTER 3
MATERIALS AND METHODS

1. Chemicals and reagents

Chemicals and reagents used throughout this study were molecular biology grade, analytical grade or tissue culture grade which were purchased from Sigma-Aldrish (USA), Boehringer Manheim (Germany), Fluka (USA), Merck (Germany), and *Phyto*Technology Laboratories (USA). All of them were absolute ethanol, acetosyringone (3,5-dimethoxy-4-hydroxy-acetophenone), agarose, alpha-Naphthaleneacetic acid (NAA), ammonium acetate, ammonium chloride, 6-Benzylaminopurine (BA), bromophenol blue, 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid cyclohexylammonium salt (X-Gluc), calcium chloride, carbenicillin, cassamino acid, cefotaxime, chloform, CHU (N6) Basal salt mixture (Sigma), clorox, 2,4-Dichlorophenoxyacetic acid (2,4-D), diethylpyrocarbonate (DEPC), *N*,*N*'-dimethylformamide (DMF), dimethylsulfoxide (DMSO), disodium ethylenediaminetetraacetic acid (EDTA), ethidium bromide, gelrite, glycerol, glycine, hexadecyl trimethyl ammonium bromide (CTAB), hydrochloric acid, hygromycin B, hydroxyquinoline, isopropanol, kinetin, phenol, magnesium chloride, magnesium sulfate 2-mercaptoethanol, myo-inositol, MS salts (Sigma), nicotinic acid, potassium acetate, potassium chloride, potassium ferricyanide, potassium forrocyanide, L-proline, pyridoxine hydrochloride, rifampicin, sodium chloride, sodium dodecyl sulfate (SDS), sodium hydroxide, sodium phosphate, sorbitol, spectinomycin, spermidine free base, sucrose, Thidiazuron (TDZ), thiamine, Tris (hydroxymethyl) aminomethane, Triton x-100 and Tween 20.

Molecular biology products were purchased from Boeringer Manheim (Germany), Invitrogen (USA), New England Biolabs (USA), Promega (USA). Bacteriological media were purchased from DIFCO (USA). The 100-bp and 1-kb

molecular weight DNA ladders were obtained from Invitrogen (USA). DNA-modifying enzymes were obtained from New England Biolabs (USA). *Tag* DNA polymerase I, 10x PCR buffer and $MgCl_2$ were obtained from Promega (USA). The access RT-PCR system was obtained from Promega (USA). TRIZOL® Reagent was obtained from GIBCO (USA). Hybond™-N^+ was purchased from Amersham (UK). ^{32}P α-dCTP was obtained from Amersham Biosciences (UK). Rediprime DNA labelling kit and X-ray films were obtained from Kodak (Australia).

2. Plant materials

2.1 Tobacco Plants

Nicotiana tabacum cv. Samson NN. was used for *Agrobacterium*-mediated transformation and particle bombardment. Seeds were surface sterilized for 10 min in 10% (v/v) clorox, washed three times in sterile water and germinated on Murashige-Skoog (MS) medium (Appendix B) (179) at 26°C under a 16-h photoperiod for 3-4 weeks.

2.2 Rice plants

Two subspecies of rice, *Oryza sativa* ssp. *japonica* cv. Taichung and *Oryza sativa* ssp. *indica* cv. KDML105 were used as plant models for *Agrobacterium*-mediated transformation and particle bombardment. Rice seeds of *japonica* were kindly provided by Prof. Junji Yamaguchi (Division of Biological Science, Graduated school of Science, Hokkaido University, Sapporo, Japan), whereas seeds of *indica* rice were kindly provided by Pathum Thani Rice Research Center, Rice Research Institute and Office of Biotechnology Research and Development, Sirindhorn Plant Genetic Resources Building, Department of Agriculture, Thanya Buri, Pathum Thani, Thailand.

3. Bacterial culture and stock culture maintenance

Bacteria, *E. coli* DH5α, were grown either in liquid LB medium (Luria-Bertani: 1% tryptone, 0.5% yeast extract and 1% sodium chloride; pH 7.0) or on solid medium (Luria-Bertani: 1% tryptone, 0.5% yeast extract, 1% sodium chloride, 1.5% bactoagar; pH 7.0) supplemented with appropriate amount of antibiotics and incubated at 37°C for 16 h. The bacterial suspension or plate could be kept at 4°C for 1-2 weeks for routine work.

Five strains of *Agrobacterium tumefaciens,* AGL1, AGL1 harboring pWBVec10a (Fig.8), AGL1 harboring pCAMBIA1301 (Fig.9), EHA105 harboring pCAMBIA1301 and EHA101 were used through this research. All of them were grown on either liquid YEP medium (1% peptone, 0.5% yeast extract and 1% sodium chloride; pH 7.0) or solid medium (1% peptone, 0.5% yeast extract and 1% sodium chloride, 1.5% bactoagar; pH 7.0) contained with appropriate antibiotics and incubated at 28°C for 2-3 days.

The concentration of antibiotics was 25 mg/l for rifampicin, 50 mg/l for kanamycin and 50 mg/l for spectinomycin. To prevent the contamination of other bacteria, rifampicin was added in all *Agrobacterium* cultured media.

For frozen stock cultures, bacterial suspension was grown at 37°C 16 h in LB for *E.coli* or 28°C 2-3 days in YEP for *Agrobacterium*, shaking at 200-250 rpm to reach the exponential growth phase. A 500 µl aliquot of the cell cultures was mixed with 500 µl of steriled 40% (v/v) glycerol for a final concentration of 20% (v/v), mixed and kept at -80°C in microcentrifuge tubes.

The genotypes of all bacteria used in this research were shown in table 2.

Table 2. Genotype of bacterial strains used through this research

Strain	Organism	Genotype
DH5-α	*E.coli*	deoR, endA1, gyrA96, hsdR17 (rk^-mk^+), recA1, relA1, supE44, thi-1,F^- (180)
EHA101	*A.tumefaciens*	C58 pTiBo542, T-region::aph, Km (R) (181)
EHA105	*A.tumefaciens*	C58 pTiBo542, T-region::aph, Km (S) (EHA105 is a Km (S) derivative of EHA101) (182)
AGL1	*A.tumefaciens*	C58 pTiBo542, recA::bla, T-region deleted Mop(+), Cb (R) (AGL1 is an EHA101 with the T-region deleted, which also deletes the aph gene) (183)

Types

Bo542	leucinopine, succinamopine, agropine type, vir weaker than A281
C58,T37	nopaline types
A281	succinamopine, leucinopine, agrocinopine

Antibiotics

Km (R)	Kanamycin resistant
Km (S)	Kanamycin sensitive
Cb (R)	Carbenicillin resistant

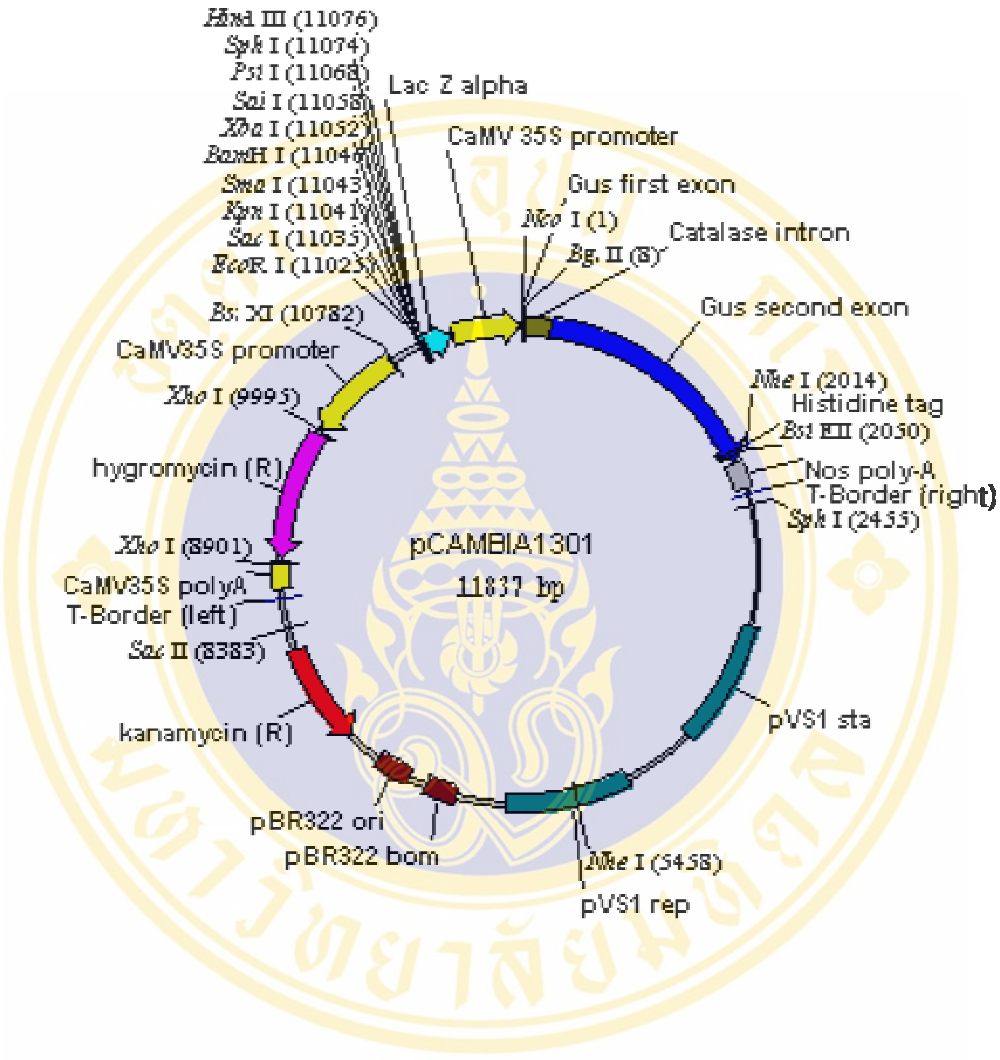

Figure 8. Schematic diagram of the plasmid pCAMBIA1301

Within the T-DNA region of this plasmid contains the β-glucuronidase (*gus*) gene with inserted catalase intron as a marker gene and the hygromycin *(hpt)* resistant gene as a selectable gene; both of them are driven by CaMV 35S promoter (182).

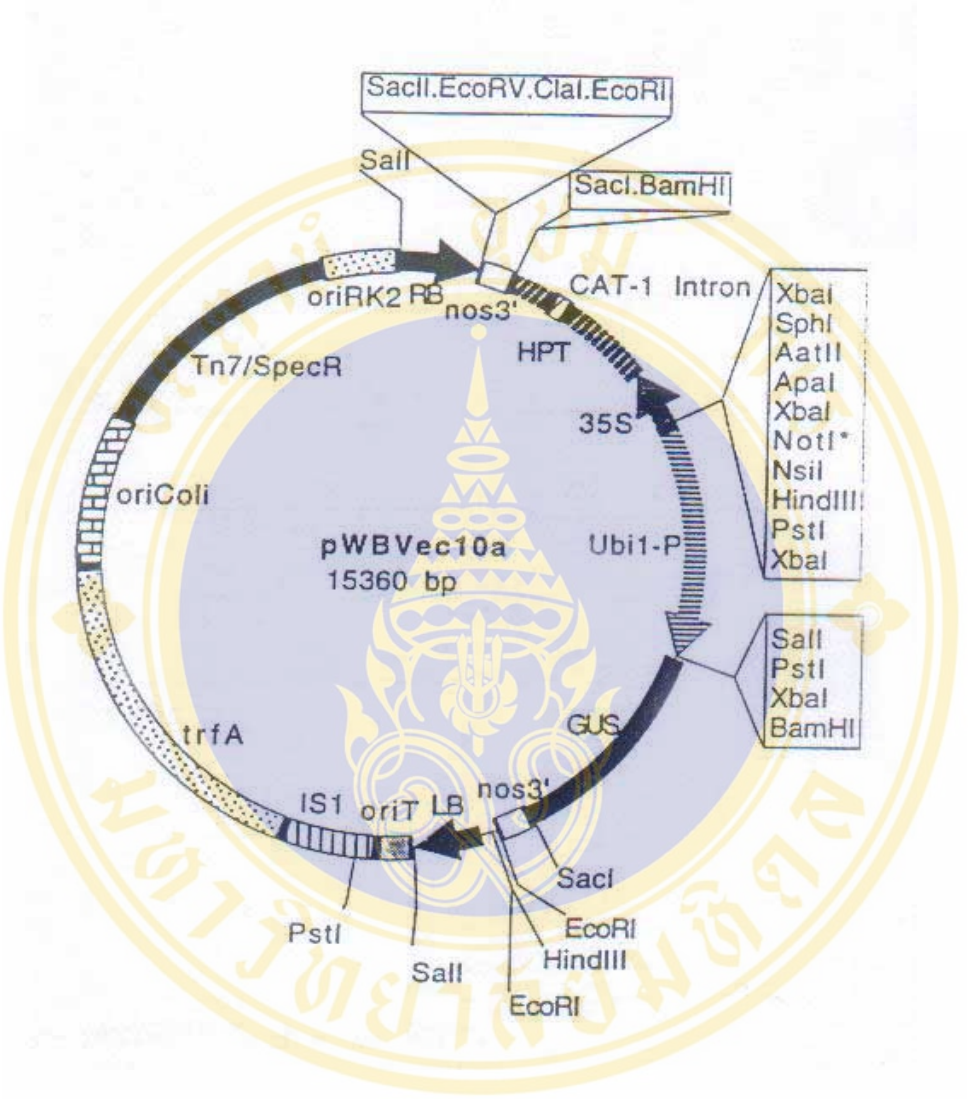

Figure 9. Schematic diagram of the plasmid pWBVec10a

The T-DNA region of this plasmid consists of the β-glucuronidase (*gus*) gene with inserted catalase 1-intron as a marker gene, driven by the rice ubiquitin promoter and the hygromycin *(hpt)* resistant gene as a selectable gene, driven by CaMV 35S promoter (184).

4. Plasmid DNA technology

4.1 Introduction of plasmid DNA into *E.coli* cells

4.1.1 Preparation of competent *E. coli* cells

The *E. coli*, DH5α, competent cells were prepared by following the method described by Sambrook (185) with some modifications as followed. *E. coli* strain DH5α was streaked on LB agar and incubated overnight at 37°C. A single colony was selected, incubated in LB broth at 37°C and shaked at 225-250 rpm for 16 h, respectively. A 1.5 ml of culture cells was transferred into 40 ml LB broth in a 125 ml Erlenmeyer flask and shaked at 37°C for 2 h. The culture was transferred to centrifuge tube and spun down at 4°C, 7,000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 10 ml of 100 mM $CaCl_2$ and mixed by vortex mixer. The mixture was placed on ice for 20 min and centrifuged for 5 min at 7,000 rpm at 4°C. The supernatant was discarded and the pellet was gently resuspended in 1 ml of 100 mM $CaCl_2$. The cells were placed on ice and subsequently dispensed in 100 µl aliquot each into chilled and steriled microcentrifuge tubes. A 100 µl steriled 40 % glycerol was added to the competent cells and mixed well. The cells were instantly frozen using liquid nitrogen and stored at -80°C until used.

4.1.2 Transformation of *E.coli*

Transformation was performed according to the method described by Sambrook (185) with some modifications. A 100 µl of competent cell aliquot was thawed on ice, gently mixed with plasmid DNA (about 10 µg) by stirring with the pipette tip. The tube was incubated on ice for 45 min. Afterwards the transformation vial was heated in a 42°C water bath for exactly 2 min and placed on ice. Then 500 µl of LB broth (Appendix B) were added and the tube was shaken horizontally at 37°C for 1 h. Aliquots of 100, 200, 300 µl were spreaded on separated LB agar plates containing appropriate antibiotics. The plates were placed at 37°C for at least 18 h.

4.2 Introduction of plasmid DNA into *Agrobacterium tumefaciens*

4.2.1 Preparation of competent *A. tumefaciens* cells

Agrobacterium was streaked on YEP agar containing 25 mg/l rifampicin and incubated 2-3 days at 28°C. The single colony was selected, incubated in 3 ml of YEP broth containing 25 mg/l rifampicin and shaked at 200-250 rpm for 24 h at 28°C. Then 1 ml of bacterial suspension was resuspended in 50 ml YEP broth containing 25 mg/l rifampicin, shaked at 200-250 rpm until A_{600} = 0.6-0.8 (about 9-10 h). The cells were selected by centrifugation at 5,000 rpm for 5 min at 4°C before gently washed with 20 ml of 10% (v/v) chilled glycerol for 3 times at 4°C. Bacterial cells were finally dispensed in 125-150 µl of 10% (v/v) chilled glycerol and then 40 µl aliquots were stored at -80°C until used.

4.2.2 Transformation of *A. tumefaciens* by electroporation

Before transformation, the 0.2 cm electroporate tube was sterilized by immersion in 70% ethanol, washed with sterile-distilled water and chilled prior to use. For transformation, the 0.05 µg/2 µl of plasmid DNA was added in 25 µl of competent cells, chilled on ice for 1 min before loaded into the electroporate tube. The mixture was transferred to the chamber of an electroporator (EC100, EC APPARATUS CORPORATION) then the exponential pulse of a voltage of 2800 V was applied to the sample. After electroporation, transformed cells were added with 1 ml of YEP medium and transferred to the microcentrifuge tube. The tube was shaken horizontally at 37°C for 1 h. Subsequently, the 100, 200, 300 µl suspensions were spreaded on YEP agar containing appropriate antibiotics and incubated at 28°C for 2-3 days.

4.3 Plasmid DNA extraction by rapid alkaline lysis method

Plasmid DNA was extracted from *E.coli* and *A. tumefaciens* by the rapid alkaline lysis method according to Sambrook (185). Bacateria DH5α was grown in 3-ml LB broth supplemented with appropriate antibiotics at 37°C with 225-250 rpm shaking for 16 h. In case of *A. tumefaciens*, the bacteria was grown in 3-ml YEP broth supplemented with appropriate antibiotics at 28°C with 225-250 rpm shaking for 2-3 days until cells reach the log phase. The 3 ml of cell culture was collected by centrifugation at 12,000 rpm for 1 min at 25°C. The pellet was resuspended in 100 µl of ice-cold solution I (50 mM glucose, 25 mM Tris-HCl; pH 8.0, 10 mM EDTA; pH 8.0), mixed by vortexing, and sit on ice for 5 min. The 200 µl of freshly prepared solution II (0.2 N NaOH, 1 % SDS) was added and immediately mixed by inverting the tube 3-4 times and then stored on ice for 5 min. This step allowed immediate breakage of the bacterial cells under alkaline condition. The 150 µl of ice-cold solution III (3M potassium acetate, 5 M glacial acetic acid) was added and mixed gently by inverting the tube for 10 s. The mixture was stored on ice for 3-5 min to denature protein content in tube, which could be seen as a white clumb of cell debris. The cell debris was removed by centrifugation at 12,000 rpm for 5 min at 4°C and the supernatant was then transferred to a new tube. Plasmid DNA in the supernatant was purified by adding 1 volume of phenol : chloroform : isoamyl alcohol (25:24:1). The tube was vigorously mixed by vortexing, followed by centrifugation at 12,000 rpm for 5 min at room temperature. The upper phase was collected and the DNA was precipitated by addition of 2 volumes of absolute ethanol and incubation for 2 min at room temperature. The DNA was collected by centrifugation at 12,000 rpm for 5 min at 4 °C, then washed once with 70 % ethanol and air-dried. The DNA was resuspended in 30 µl of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA; pH 8.0) or sterile deionized water, treated with 20 µg/ml of RNase A (prepared by dissolving 10 mg of RNase A in 1 ml of 10 mM Tris-HCl pH 7.5 and 15 mM NaCl, boiled for 15 min and then stored at -20°C) at 65°C for 10 min and stored at -20°C.

4.4 Digestion with restriction endonucleases

Plasmid DNA digestion or modification with various restriction and modifying enzymes for this study was performed using the buffer provided with the enzymes under the conditions recommended by the manufacturers. When digestion of DNA with two different enzymes was required, in the case where reaction conditions (buffer and temperature) were compatible, both enzymes were added simultaneously. However, when the reaction conditions were incompatible, in the general the low salt reaction was performed before and an ethanol precipitation was performed before the second enzymatic digestion.

4.5 Agarose gel electrophoresis

Electrophoresis in agarose gel was performed to separate, visualize and purify desired DNA fragments. The concentration of agarose gel was various (0.8-2.0%), according to the size of DNA fragment. The agarose gel for electrophoresis was prepared with suitable volume of 1X TAE buffer (0.04 M Tris acetate, 0.002 M EDTA; pH 8.0). The agarose particle was completely dissolved by heating, allowed to cool down and poured into a gel tray, which was set on gel caster. A horizontal type electrophoresis chamber (GelMate 2000) was routinely used for DNA analysis. DNA solution was mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) and loaded into a well of gel in a submarine condition. Two standard markers, 100 bp and 1 kb DNA ladder (Invitrogen) were used to estimate the size of DNA fragment on agarose gel electrophoresis. Electrophoresis was performed in 1X TAE buffer, at constant voltage of 70-100, depend on the percentage of the agarose gel. Agarose gel was then stained with 0.5 μg/ml of ethidium bromide solution for 10-15 min and DNA fragment was visualized on a UV transilluminator and photographed using Gel documentation system (GENE GENIOUS Bio Imaging System, software GeneSnap from SYNGENE).

5. *Agrobacterium*-mediated transformation in tobacco

The method of leaf disk transformation was followed by Horsch *et al.* (186) with some modifications. In this experiment, we used *Agrobacterium* strain EHA105 harboring the plasmid pCAMBIA1301, for tobacco transformation. The bacteria were grown in 30 ml of YEP medium supplemented with 25 mg/l rifampicin and 50 mg/l kanamycin at 28°C with 200 rpm shaking until OD = 0.3 - 0.4 at λ-600 nm. Tobacco leaves were cut into small pieces (0.8×0.8 cm). The primary vein of leaves or necrotic areas was avoided. Eighty disks were immersed in bacterial suspension about 1-2 min and co-cultivated on RMOP medium (Appendix B) (187) at 26°C under a 16-h photoperiod for 3 days. The bacteria were removed from plant tissues by rinsing 3 times with 500, 250 and 250 mg/l carbenicillin, respectively, before blotted dry on filter papers. Ten pieces of them were selected in order to check the transient expression by histochemical analysis as described in 10. Other tissues were placed on RMOP medium containing 50 mg/l hygromycin B and 250 mg/l carbenicillin for selection of putative transgenic plants. It should be noted that, the tissues had to be transferred to the fresh medium every week to prevent the bacterial contamination. Eight weeks later, transgenic shoots regenerating from the resistant calli were transferred to MS medium containing 50 mg/l hygromycin B and 250 mg/l carbenicillin for further development to get the whole plants. The step of gene transformation was shown in Fig. 10.

6. *Agrobacterium*-mediated transformation in callus of *japonica* rice (cv. Taichung)

Rice seeds were dehusked and surface sterilized in 95% ethanol for 30 s and 40% clorox for 30 min. Seeds were rinsed 5 times with sterile distilled water and placed on N6D medium (Appendix B) for callus induction. After the seeds were cultured under a dimming light at 26°C for 2 weeks, developing calli were separated from endosperms and precultured on the same medium for 3 days. *Agrobacterium* strain EHA105 harboring the plasmid pCAMBIA1301 was grown on AB medium (188) at 28°C for 3 days, then the bacterial colonies were collected and suspended in AAM medium (189)

(Appendix B) containing 10 mg/l acetosyringone with OD = 0.6-1.0 at λ-600 nm. The precultured calli were immersed in the bacterial suspension for 30 s, blotted on tissue papers and co-cultivated on 2N6-AS medium (Appendix B) for 2 days. After that, the calli were washed with 250 mg/l carbenicillin for 3 times, 5 min each in order to remove the bacteria. Some of transformed calli were incubated in X-Gluc solution (see 10.) for observation of transient expression. To select the transgenic calli, the calli were cultured on N6D containing 250 mg/l carbenicillin and 50 mg/l hygromycin B for 3 weeks. The resistant calli were transferred to MS-NK medium (Appendix B) for approximately 4-5 weeks for plant regeneration. Finally, the regenerated plants were transferred to MS-HF medium (Appendix B) for root induction. The step of transformation system was shown in Fig. 11.

7. *Agrobacterium*-mediated transformation in shoot apical meristem of *indica* rice (KDML105)

7.1 Multiple shoot regeneration from shoot apical meristem culture

Prior to perform the transformation experiment, the tissue culture system of *indica* rice (KDML105) has been established in collaboration with the Office of Biotechnology Research and Development, Department of Agriculture, Thailand. Mature seeds were dehusked, sterilized in 95% ethanol for 30 s and 40% clorox for 30 min, rinsed 5 times with sterile distilled water and placed on MS medium supplemented with 0, 1, 2, 4, 6, 8 mg/l thidiazuron (TDZ). Three days after incubation at 27°C under 16-h photoperiod, germinated seedlings were isolated from endosperms. Roots were then removed carefully and completely by cutting out a shoot tip, leaving about 1-mm-long shoot with the thick basal portion. The dissection of the shoot tip would stimulate the formation of multiple shoots. After subcultured on the fresh medium for 3 days, the longest was cut out, leaving about 2-3-mm-long shoots and transferred to the fresh medium. Two weeks later, the number of regenerated shoots/meristem was recorded. Multiple shoots were separated from each other and transferred to MS medium without the growth regulator for root formation. The step of shoot apical meristem transformation was shown in Fig. 12.

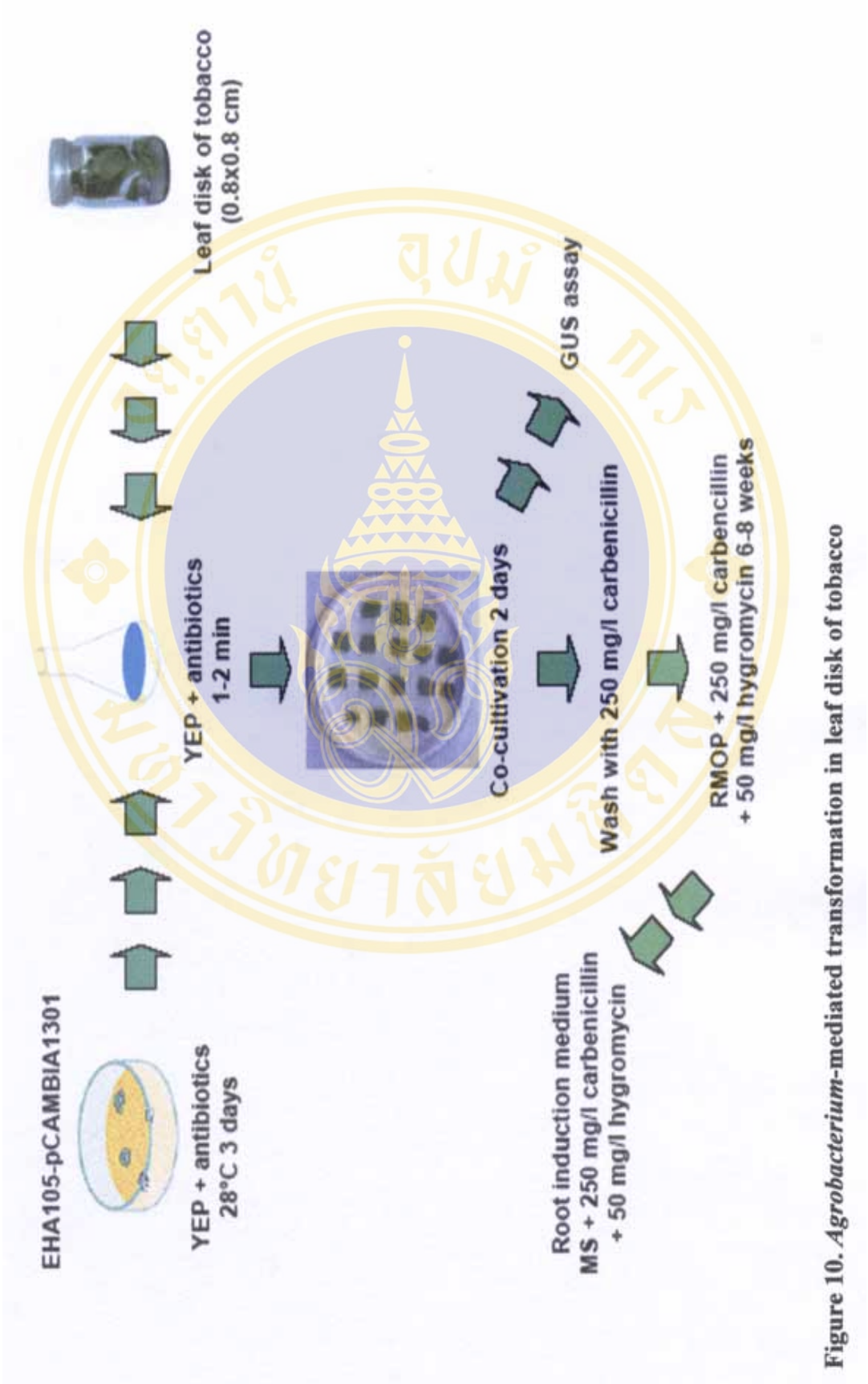

Figure 10. *Agrobacterium*-mediated transformation in leaf disk of tobacco

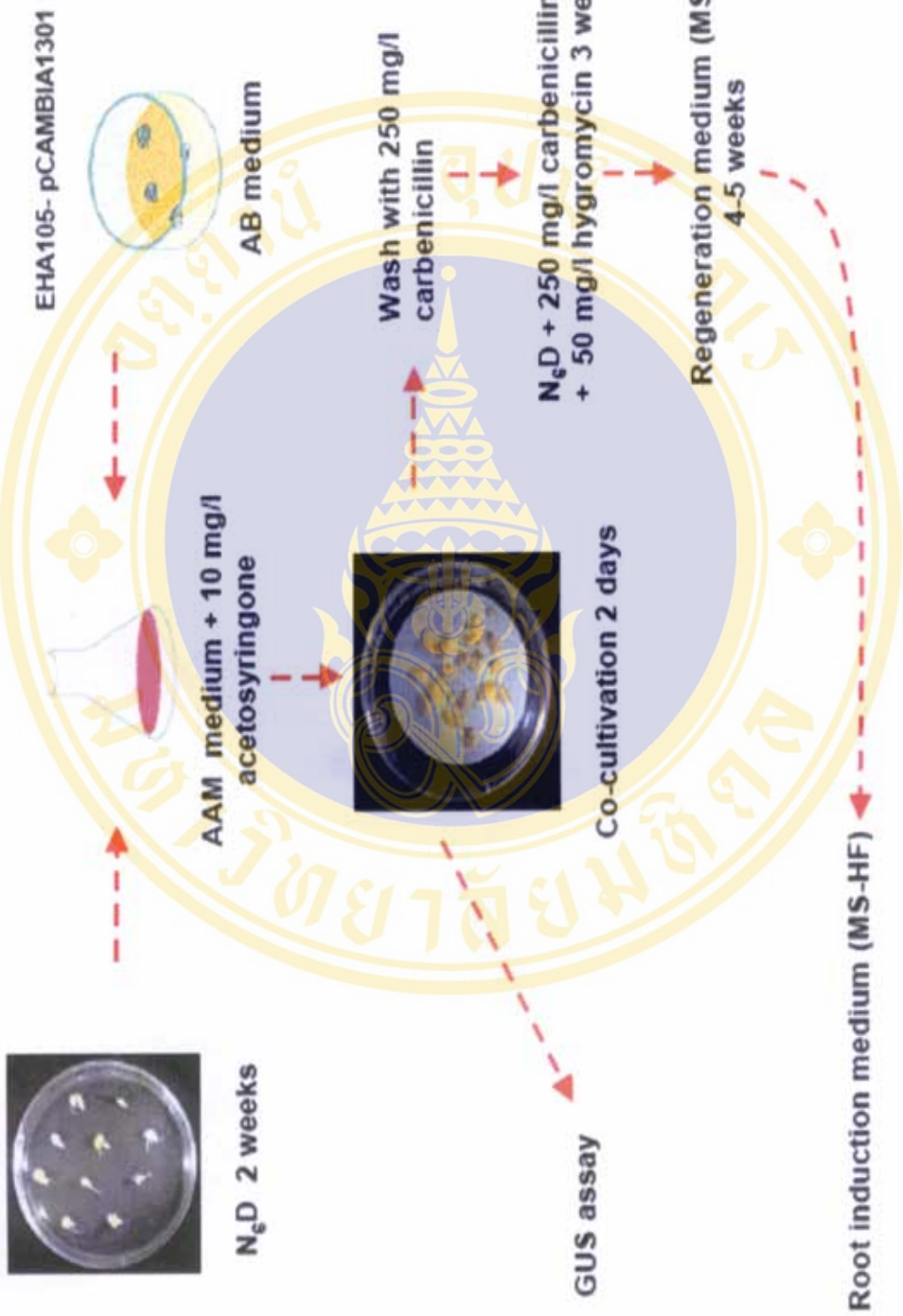

Figure 11. *Agrobacterium*-mediated transformation in callus of *japonica* rice

7.2 Study the effect of hygromycin B, cefotaxime and carbenicillin on multiple shoot regeneration

Mature seeds were dehusked, sterilized in 95% ethanol for 30 s and 40% clorox for 30 min, rinsed 5 times with sterile distilled water and placed on MS medium supplemented with 4 mg/l TDZ. After 3 days, shoot apical meristems were isolated from endosperms as described in 7.1 and placed on MS-TDZ supplemented with 250 mg/l cefotaxime + 0, 50, 75, 100, 150 mg/l hygromycin B and 250 mg/l carbenicilin + 0, 50, 75, 100, 150 mg/l hygromycin B. The viability of regenerated shoots was observed at 1, 2, 3 and 4 weeks.

7.3 Study the viability of *Agrobacterium* on antibiotic-supplemented medium

In order to study the resistance of *A. tumefaciens* to antibiotics related to the transformation experiment, *Agrobacterium* strain AGL1, AGL1 harboring pWBVec10a, AGL1 harboring pCAMBIA1301, EHA105 harboring pCAMBIA1301 and EHA101 were grown on YEP medium containing 25, 50 mg/l rifampicin, 250 mg/l cefotaxime, 250 mg/l carbenicillin, 50 mg/l hygromycin B, 50 mg/l spectinomycin and 50 mg/l kanamycin at 28°C for 2 days. The survival colonies of the bacteria were observed.

7.4 Study the effect of acetosyringone on gene transformation efficiency

EHA105-pCAMBIA1301 was cultured on YEP medium supplemented with 25 mg/l rifampicin and 50 mg/l kanamycin, at 28°C for 2 days. A bacterial colonies were collected and suspended in YEP medium until OD_{600} = 1.0, followed by incubation at 28°C 250 rpm with shaking for 1 h. To evaluate the effect of acetosyringone, bacterial suspension was further cultured under the same condition with or without 200 μM acetosyringone for 2 h before transformation. Rice seeds were dehusked, sterilized in 95% ethanol for 30 s and 40% clorox for 30 min, rinsed 5 times with sterile distilled water and precultured on MS medium supplemented with 4 mg/l TDZ in order to induce multiple shoot formation. After 3 days, shoot apical meristems were isolated

from endosperms as described above. Then, immersed in bacterial suspension for 1 min and placed on MS-TDZ supplemented with or without 200 μM acetosyringone. The tissues were co-cultivated at 27°C under a 16-h photoperiod for 3 days and then transient expression was detected by histochemical analysis as described in 10. The experiment was repeated 5 times and 25 explants were used in each experiment.

7.5 Study the efficiency of gene transformation in various sonication times

EHA105-pCAMBIA1301 was cultured as described above and supplemented with 200 μM acetosyringone for 2 h before transformation. Shoot apical meristems were immersed in the bacterial suspension and sonicated with 4 treatments (0, 5, 10 and 20 s) before co-cultivated on MS containing 4 mg/l TDZ and 200 μM acetosyringone. After 3 days of co-cultivation, transient expressions were analyzed using histochemical analysis. The experiment was repeated 4 times and 25 explants were used in each treatment. Means of the number of GUS-expressing seedlings in each treatment was calculated and analyzed by ANOVA. Duncan's multiple range test (DMRT) at $p = 0.05$ was used to compare the means of each treatment.

7.6 Establishment of stable transformation in shoot apical meristem

EHA105-pCAMBIA1301 was cultured as described above and supplemented with 200 μM acetosyringone for 2 h before transformation. Shoot apical meristems were immersed in the bacterial suspension and sonicated at 10 s, then co-cultivated on MS containing 4 mg/l TDZ and 200 μM acetosyringone. After 3 days of co-cultivation, shoot apical meristems were washed with 250-300 mg/l carbenicillin 5-10 min for 3 times. A shoot tip was removed, leaving around 2-3-mm-long shoots with the thick basal portion and transferred to MS-TDZ contained 25 mg/l hygromycin B and 250 mg/l carbenicillin. Plant tissues were transferred to fresh medium every week. Brown and necrosis tissues from the effect of hygromycin B were removed from the explants. Regeneration of resistant shoots could be observed within 2-3 weeks on the

selective medium. Finally, the resistant shoots were transferred to MS medium containing 25 mg/l hygromycin B and 250 mg/l carbenicillin in order to get the whole rice plants.

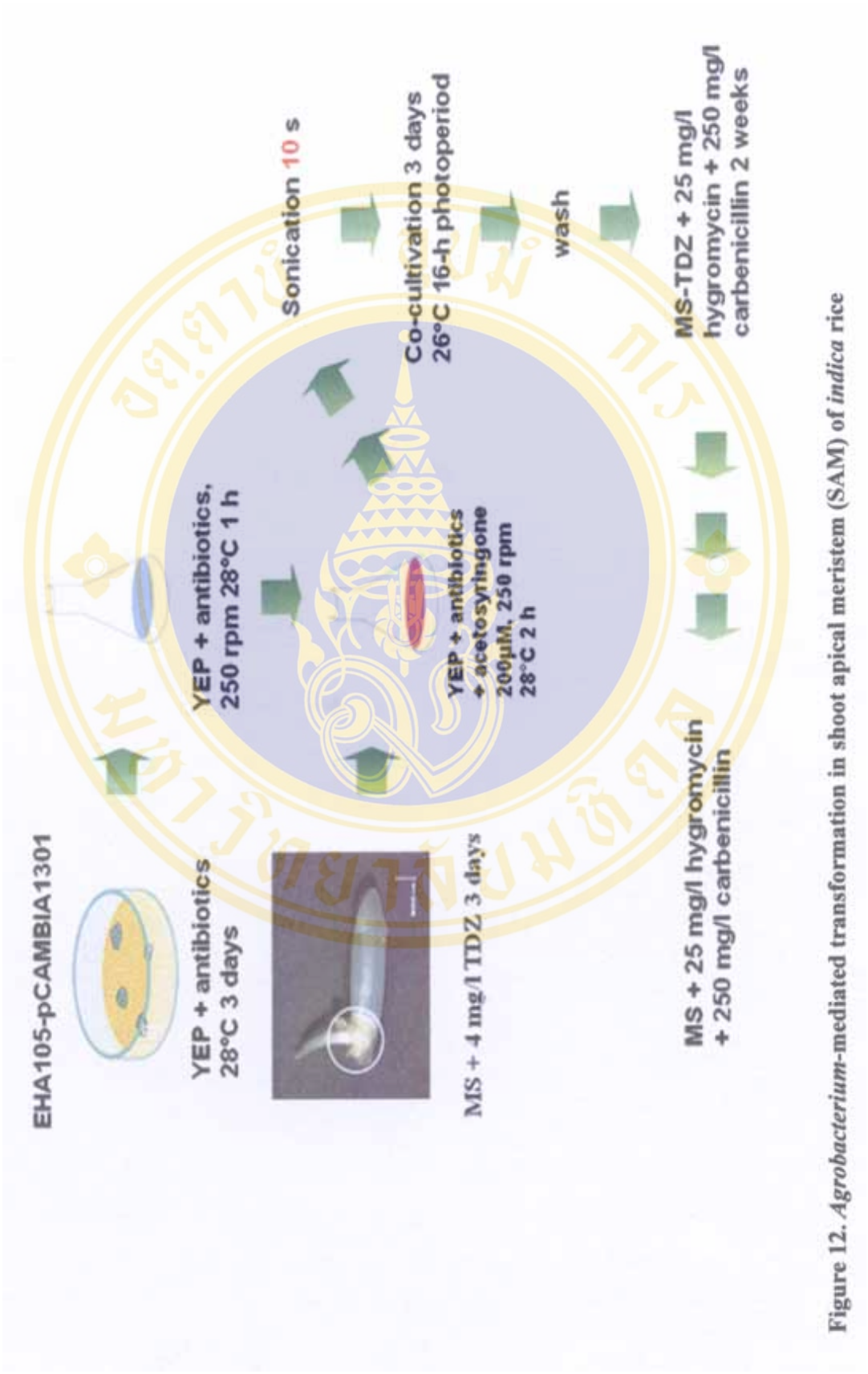

Figure 12. *Agrobacterium*-mediated transformation in shoot apical meristem (SAM) of *indica* rice

8. Particle bombardment in tobacco

8.1 The bombardment process

The biolistic PDS-1000/He system (BIO-RAD) (Fig. 13) uses high pressure helium, released by a rupture disk, and partial vacuum to propel a macrocarrier sheet loaded with millions of microscopic tungsten or gold microcarriers toward target cells at high velocity. The microcarriers are coated with DNA or other biological material for transformation. The macrocarrier is halted after a short distance by a stopping screen. The DNA-coated microcarriers continue traveling toward the target to penetrate and transform the cells. The launch velocity of microcarriers for each bombardment is dependent upon the helium pressure (rupture disk selection, the amount of vacuum in the bombardment chamber, the distance from the rupture disk to the macrocarrier, the macrocarrier travel distance to the stopping screen and the distance between the stopping screen and target cells (Fig. 14).

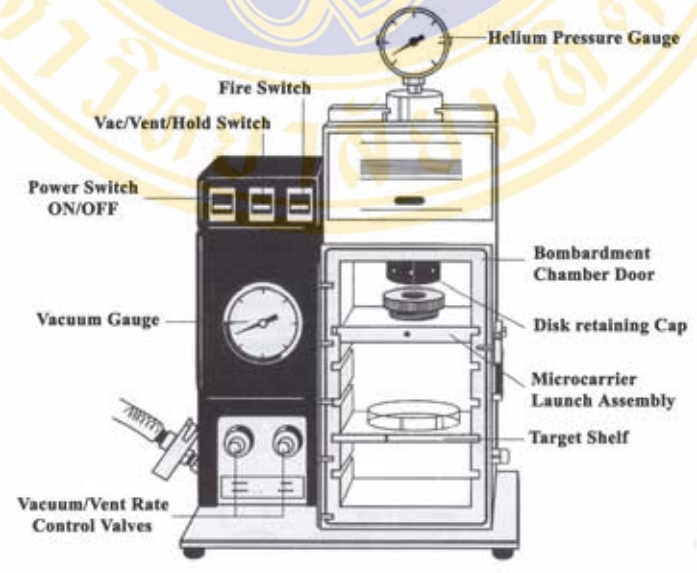

Figure 13. Front view of PDS-1000/He unit (BIO-RAD)

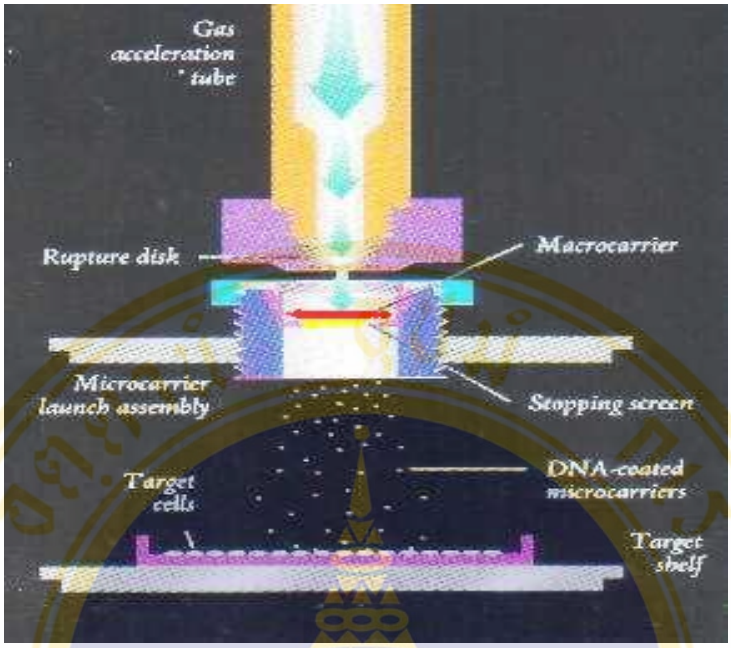

Figure 14. The particle bombardment process

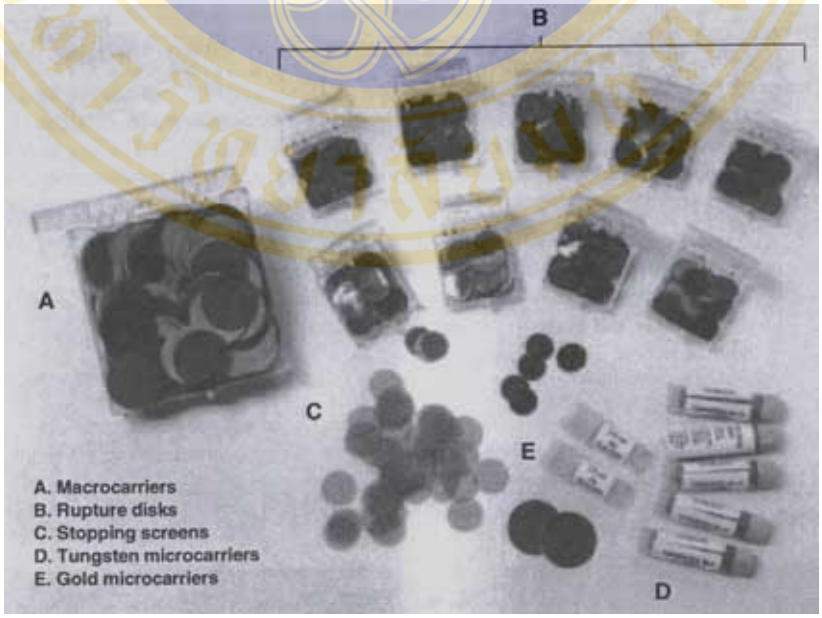

Figure 15. Consumable for the PDS-1000/He instrument

8.2 Preparation of plant material

Tobacco leaves from tobacco cultured on MS medium (Appendix B) for 3-6 weeks were cut into pieces (0.8×0.8 cm). Then, the explants were placed abaxial side up at the center of the plates on top of a sterile filter disks on an RMOP medium (Appendix B) (187) thin plates and precultured overnight at 26°C in a culture room.

8.3 Preparation of microcarriers

The microcarrier preparation and procedure for coating DNA onto microcarriers were developed by Sanford (190). The whole procedure was carried out at room temperature. To prepare microcarriers, 60 mg of 1-µm-gold microparticles were washed with 1 ml of freshly prepared 70% ethanol. The microparticles were thoroughly mixed with ethanol by vortexing for 3-5 min and then incubated for 15 min. The microparticles were collected by spinning down for 5 s and the liquid was carefully discarded. Ethanol was completely removed by washing pellet with steriled water 3 times as followed. The pellet was mixed with 1 ml steriled water, thoroughly washed by vortexing for 1 min, and spinning down for 2 s to collect the pellet. After washing for 3 times, 1 ml of 50% glycerol was added to bring the microparticle concentration to 60 mg/ml.

8.4 Coating of DNA on microcarriers

For preparation of 6 bombardments, the washed gold particles in 50% glycerol (from 8.3) were either vortexed for 5 min on a platform vortexer or sonicated to resuspend and disrupt agglomerated particles. After that, 50 µl (30 mg) of microcarriers was transferred to a new 1.5 ml microcentrifuge tube. While vortexing vigorously, 5 µl of plasmid DNA (1µg/µl), 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine (free base, tissue culture grade), were added respectively and the tube was continuously vortexed for 2-3 min. The microcarriers were allowed to settle for 1 min, spun down for 2 s to precipitate the microcarriers and then the liquid was discarded. The pellet was respectively washed with 140 µl of 70% ethanol, spun down for 2 s

and the liquid was removed and discarded followed by the addition of 48 µl of 100% ethanol to resuspend the pellet. In the last step, the resuspended microcarriers were gently mixed by flicking the tube and vortexing at low speed for 2-3 s.

8.5 Performing a bombardment

Prier to perform the bombardent, a microcarrier launch assembly, a rupture disk retaining cap and stopping screens were sterilized by autoclaving at 121°C 15 min. The macrocarriers were also assembled in macrocarrier holders and autoclaved. After coating of DNA on microcarriers, 8 µl aliquots of the gold microcarriers were loaded and spread evenly, 1 cm of the macrocarrier in the center, using a pipette tip (Fig. 16A). Ethanol was allowed to evaporate within 10 min to leave the DNA-coated microcarriers adhering to the macrocarrier. The chamber of the PDS-1000/He system was cleaned with 70% ethanol and the helium gas pressure in tank was set to 200 psi over the selected rupture disk burst pressure. In this experiment, the regulator was set to 1,300 psi because the bombardment was performed with a 1,100 psi rupture disk. The rupture disk, which was cleaned with 70% isopropanol, was loaded in the rupture disk retaining cap (Fig. 16B) and the retaining cap was placed at the gas acceleration tube within the bombardment chamber (Fig. 16C). A stopping screen was placed on the stopping screen support inside the microcarrier launch assembly (Fig. 16D) and the macrocarrier/macrocarrier holder was placed on the fixed nest of the microcarrier launch assembly so that the microcarriers (on macrocarrier/macrocarrier holder) were face down towards the stopping screen. The macrocarrier coverlid was placed on the assembly and screwed until snug. The microcarrier launch assembly was then placed in the top slot inside the bombardment chamber. A petri dish containing the tobacco leaves was placed on the target shelf at the target distance of 9 cm and the door was closed. The bombardment was performed according the instruction of BIO-RAD using the vacuum level at 28 inches Hg.

Figure 16. Performing a bombardment

(A) Loading of DNA-coated microcarriers onto a macrocarrier/macrocarrier holder, (B) Rupture disk insertion into recess of retaining cap, (C) Proper torque applied to retaining cap with torque wrench, (D) Removal/replacement of macrocarrier coverlid with assembled fixed nest, (E) Macrocarrier holder (with macrocarrier properly inserted) was inverted and placed atop fixed nest.

8.6 Selection and regeneration of transgenic plants

After performing the bombardment, plant tissues were transferred to fresh RMOP medium without filter paper and cultured at 26°C under a 16-h photoperiod for 2 days. The bombarded tobacco leaves were cut into small pieces (0.8x0.8 cm) and placed on RMOP medium containing 50 mg/l hygromycin B so as to select the transformants. Besides, some of them were tested for the transient expression by histochemical analysis as described in 10. The resistant shoots were regenerated on the selective medium after cultured for 5-8 weeks. The putative transgenic shoots were then transferred to MS medium containing 50 mg/l hygromycin B for root induction. Particle bombardment process in tobacco was summarized in Fig. 17.

9. Particle bombardment in shoot apical meristem of *indica* rice (cv. KDML105)

Rice seeds were dehusked, sterilized in 95% ethanol for 30 s and 40% clorox for 30 min. Seeds were rinsed 5 times with sterile distilled water and placed on MS medium supplemented with 4 mg/l TDZ to induce multiple shoots formation. After 3 days, shoot apical meristems were isolated from endosperms as describe above, 50 apices were placed on the same medium in a middle of the plates and precultured for 4 h before bombardment. Gold particles were washed and coated with the plasmid pCAMBIA1301 as described above. The particle bombardment parameters in this experiment were performed in table 3. After bombardment, rice tissues were cultured on the same medium for 2 days and then the number of GUS-expressing seedlings was counted as described in 10.

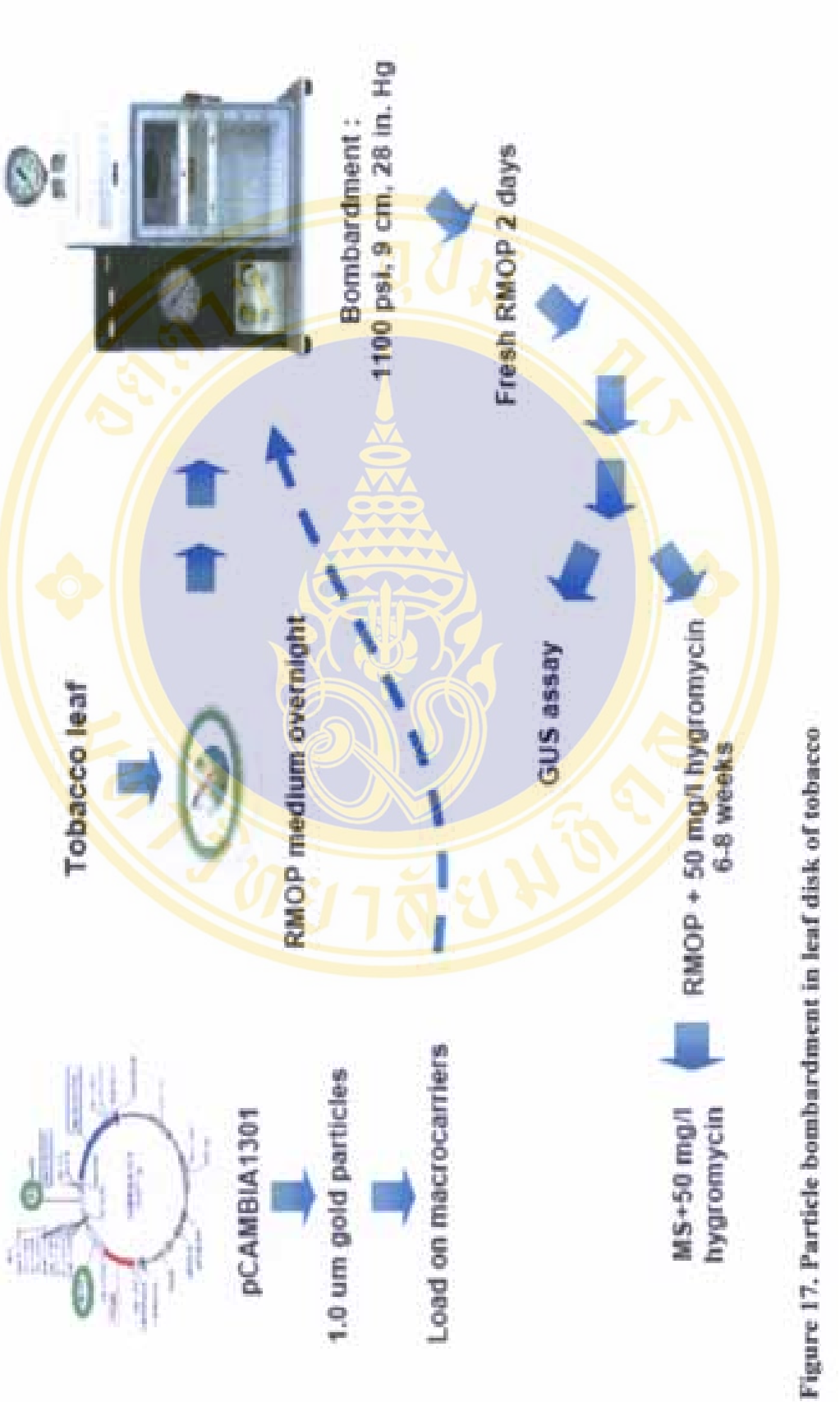

Figure 17. Particle bombardment in leaf disk of tobacco

Table 3. Tested parameters for particle bombardment in shoot apical meristem of KDML105

Parameter	Tested Condition
Plasmid DNA (pCAMBIA1301)	1 μg/μl
Acceleration pressure	1100, 1350 psi
Target distance	9, 12 cm
Chamber vacuum pressure	28 inches Hg
Number of bombardments	1, 2

10. Histochemical Analysis

The use of the *E. coli* β-glucuronidase (GUS) system as a reporter gene in plant molecular biology has helped the analysis and interpretation of the factors mediating the regulation of gene expression. Coinciding with the use of the GUS marker gene system has been the development of a series of GUS substrates and analytical methods for use in detecting enzyme expression levels. Histochemical staining of β-glucuronidase was first reported in the early 1950s for localization of endogenous enzyme in mammalian tissue (191). The preferred substrate for localization is 5-bromo-4-chloro-3-indoyl-β-D-glicuronide, or X-Gluc. The reaction proceeds through an unstable intermediate, which then undergoes as oxidative dimerization to the intensely blue ClBr-indigo (Fig. 18). The second characteristic makes X-Gluc ideal for localization because ClBr-indigo immediately precipitates formation allowing precise cellular localization of enzymatic activity and little loss of enzyme product, ClBr-indigo, in solvents typically used during tissue processing (192).

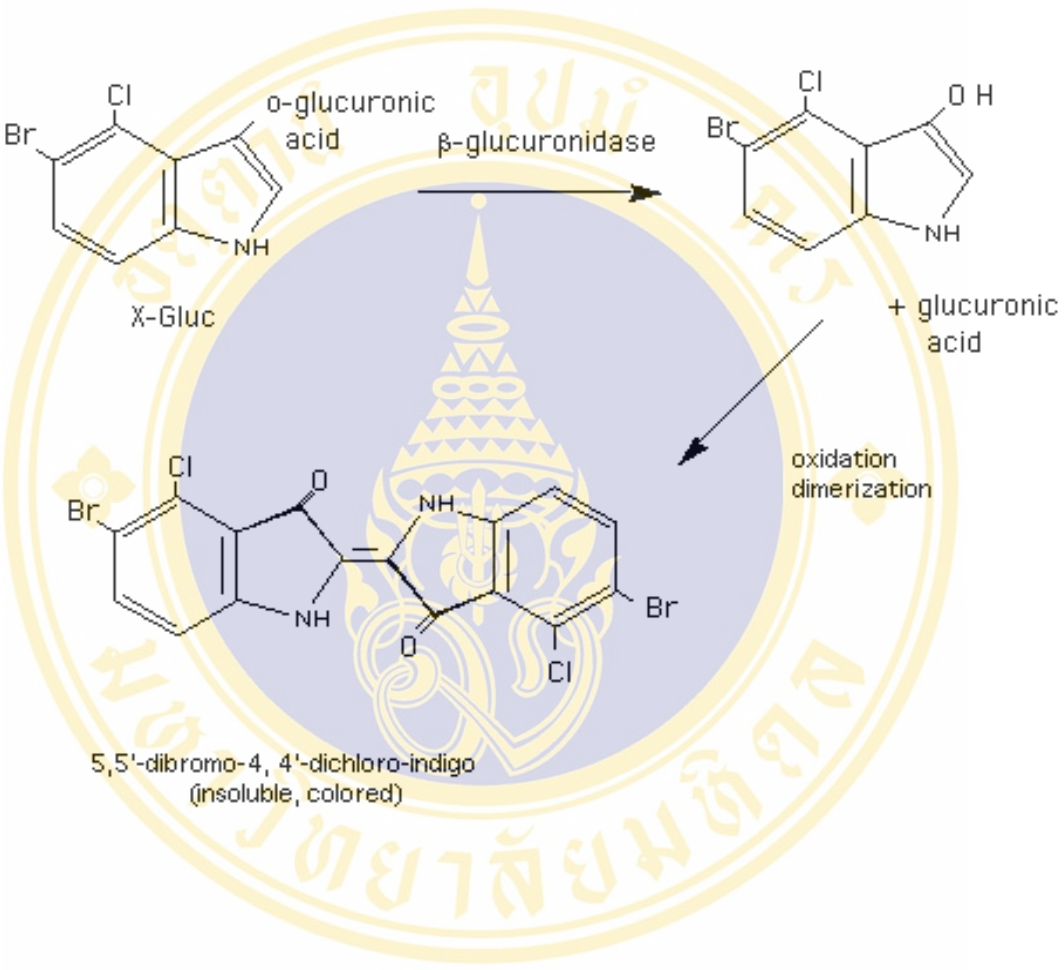

Figure 18. Cleavage of 5-5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid, or X-Gluc, produces the final insoluble precipitate dichloro-dibromoindigo (ClBr-indigo).

10.1 Reagent preparation and storage

The method of histochemical analysis in this research was developed by Jefferson *et al.* (193). The reagent mix typically contains three components: the substrate (X-Gluc), the buffer (0.1 M sodium phosphate; pH 7.0) and the oxidation catalyst (0.5 mM each potassium ferri- and ferocyanide; pH 7.0, plus 10 mM EDTA; pH 7.0) (Table 4).

The substrate, 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid or X-Gluc, was stored frozen at -20°C. Stock solution was made up as 0.02 M in N, N' dimethylformamide (DMF), aliquoted into convenient amounts and stored in microcentrifuge tubes in the dark at -20°C up to 6 months. Degradation of stock solutions could be recognized by a brown to purple color change. Certain plastics, such as polystyrene, would react with dimethylformamide and should not be used.

Table 4. The component of reagent mix for histochemical analysis (GUS staining)

Stock solution	Final concentration	Reagent mix (μg/ml)*
1.0 M $NaPO_4$ buffer, pH 7.0	0.1 M	100
0.25 M EDTA, pH 7.0	10 mM	40
0.005 M Potassium ferricyanide, pH 7.0	0.5 mM	100
0.005 M Potassium ferrocyanide, pH 7.0	0.5 mM	100
0.02 M x-Glucuronide	1.0 mM	50
10% (v/v) Triton x-100	0.1%	10
Subtotal		**400**
Distilled water		600
Final volume		**1000**

* A single large batch of reagent mix could be stored at least 6 month at -20°C

Formation of ClBr-indigo precipitates is slow, and diffusion of the soluble indoyls into neighboring cells not expressing β-glucuronidase migh also exhibit a blue coloration. Therefore, the addition of potassium ferri- and ferrocyanide to the X-Gluc solution prevent the diffusion of the indoyls by accelerating the dimerization of the colorless cleavage intermediate into the colored final product. Ferri- and ferrocyanide was freshly prepared every 2 months because the pale-yellow ferrrocyanide would turn darker yellow due to oxidation of the ferrous ion to the ferric ion.

10.2 Incubation condition

Fresh plant tissues were immersed in the reagent mix and incubated at 37°C from 1 h to overnight until the staining was satisfactory. Chlorophyll pigment was removed by several washed of 70% ethanol until the nonstained tissues were white color.

11. DNA analysis

11.1 Isolation of genomic DNA

The method described by Dellapota *et. al.* (194) with some modifications was used to prepare genomic DNA of rice and tobacco leaves. Tissues were frozen by adding liquid nitrogen and quickly ground in mortar to fine powder and tranferred to microcentrifuge tube immediately. The 600 µl of extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl), 60 µl of β-mercaptoethanol and 60 µl of 20% SDS were added to the fine powder tissues and mixed by vortex. The slurry was incubated at 65°C for 10 min. The 50-µl 5 M potassium acetate was added and mixed well. Then, the mixture was incubated at -20°C for 20 min, and subsequently centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was transferred into new microcentrifuge tube and 400 µl of ice-cold isopropanol was added before incubating at -20°C, 20 min. To collect the DNA pellet, the sample was centrifuged at 7,000 rpm for 10 min at 4°C and washed once in cold 80 % ethanol. Subsequently, the pellet was allowed to air dry at room temperature and resuspended in 20 µl of sterile deionized

water. The DNA suspension was spun at 7,000 rpm for 5 min at 4°C to remove the remaining insoluble materials. Finally, the DNA solution was transferred to another new microcentrifuge tube and treated with 20 μg/ml of RNase A at 65°C for 10 min before stored at -20°C.

11.2 PCR amplification

In order to determine the patterns of transgene integration in transgnic plants, 7 pairs of primers (1+1, 1+2, 2+2, 3+4, 5+6, 7+8, 7+9) were designed from pCAMBIA1301 using vector NTI program as described; p1+1, p1+2, p2+2 were primers for detection of transgene rearrangement with direct or inverted repeats that may be presented in the transgenic lines; p3+4 were designed for *gus* gene amplification, p5+6 for *hpt* gene amplification, p7+8 and p7+9 were designed to detect the presence of CaMV 35S promoter linked to *hpt* gene and CaMV 35S linked to *gus* gene, respectively (Fig. 19).

Primers of *actin* gene were kindly provided by Tungsuchat T., M.Sc. thesis (2001), Department of Biotechnology, Faculty of Science, Mahidol University. The sequences and PCR products of all primers were shown in table 5.

PCR amplification (35 cycles) was carried out in a 25-μl reaction containing 200 ng template DNA, 1 unit of *Tag* DNA polymerase I (Promega), 1x reaction buffer (50 mM potassium chloride, 10 mM Tris-HCl; pH 9.0 at 25°C, 0.1% Triton ® X-100), 1.5 mM $MgCl_2$, 200 μM each dNTP and 1.8 μM each primer. The PCR reactions were performed in a Perkin Elmer DNA Thermal Cycler under the condition as shown in table 6.

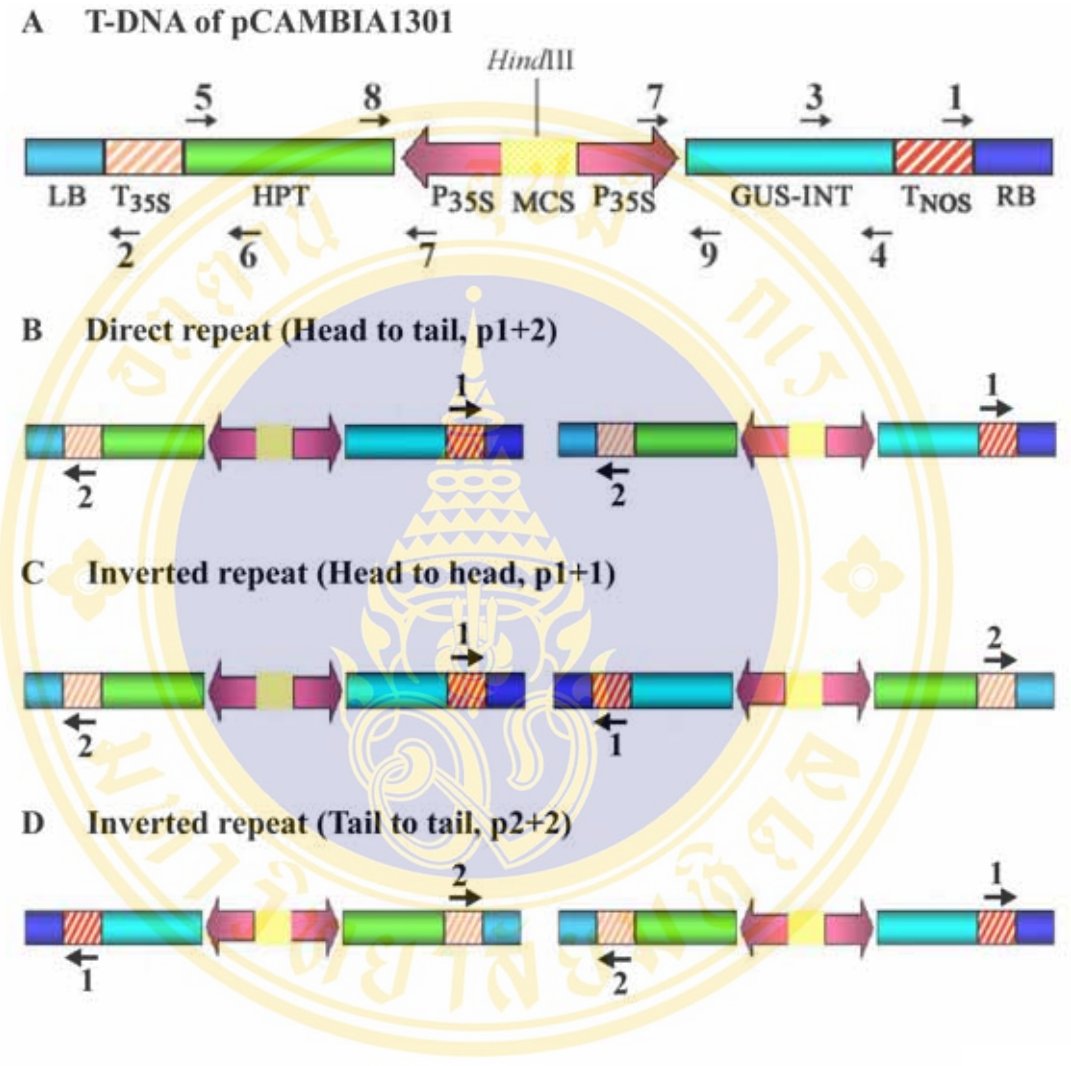

Figure 19. Schematic diagrams of possible transgene repeat arrangements in transgenic plants. (A) T-DNA of pCAMBIA1301 with left (LB) and right (RB) borders. The number 1-9 represents the different primers to be used for detection of transgene integration patterns. (B) T-DNA direct repeats in the form of head-to-tail integration. (C) Inverted T-DNA repeats in head-to-head and (D) in tail-to-tail.

Table 5. Sequences of primers for PCR amplification

Gene	Primer	Sequence	PCR product (bp)
Rearrangement of T-DNA	PCAM-1	5'TGTTGCCGGTCTTGCGATG	ND
	PCAM-2	5'TAGGGTTTCGCTCATGTGTTGAGC	
gus	PCAM-3	5'CACACCGATACCATCAGCG	425
	PCAM-4	5'AAGTTCATGCCAGTCCAGCG	
hpt	PCAM-5	5'TTGCCCTCGGACGAGTGC	373
	PCAM-6	5'TGACTGGAGCGAGGCGATG	
CaMV 35S-*hpt* (7+8)	PCAM-7	5'ATGCCTCTGCCGACAGTGG	574 (7+8)
	PCAM-8	5'TCGGTTTCAGGCAGGTCTTG	
CaMV 35S-*gus* (7+9)	PCAM-9	5'TGAGTGACCGCTTCGAAACG	699 (7+9)
Actin	Actin-F	5'TCGTACTCAGCCTTGGCAATCCAC	364
	Actin-R	5'CATGAAGACTACATACAACTCCATC	

ND = Not determine: the PCR products are depended on transgene integrations and rearrangements

Table 6. The cycling conditions of primers used in PCR amplification

Primer	PCR step				
	Denaturation	35 cycles of			Extension
		Denaturation	Annealing (Ta)	Extension	
1+1	95°C, 5 min	95°C, 1 min	53°C, 1 min	72°C, 1 min	72°C, 10 min
1+2	95°C, 5 min	95°C, 1 min	53°C, 1 min	72°C, 1 min	72°C, 10 min
2+2	95°C, 5 min	95°C, 1 min	53°C, 1 min	72°C, 1 min	72°C, 10 min
3+4	95°C, 5 min	95°C, 1 min	52°C, 1 min	72°C, 1 min	72°C, 10 min
5+6	95°C, 5 min	95°C, 1 min	54°C, 1 min	72°C, 1 min	72°C, 10 min
7+8	95°C, 5 min	95°C, 1 min	52°C, 1 min	72°C, 1 min	72°C, 10 min
7+9	95°C, 5 min	95°C, 1 min	52°C, 1 min	72°C, 1 min	72°C, 10 min
Actin	95°C, 5 min	95°C, 1 min	50°C, 1 min	72°C, 1 min	72°C, 10 min

11.3 Southern bolt analysis

Southern blot analysis was performed following the method of Sambrook (185, 193). Ten μg of DNA was cut with appropriate enzymes, run in gel electrophoresis, after that the gel was soaked in 2 successive bath of denaturation solution (1 M HCl, 0.4 N NaOH) at room temperature with constant shaking for 15 and 20 min, respectively. During the gel treatment, one piece of nylon membrane (Hybond^{TM}-N^{+}, Amersham, UK) was cut and wet in the basic denaturation solution for 5 min. The DNA was transferred by capillary onto the wet nylon membrane as followed: The gel was placed on a Whatman paper bridge, the extremely of which were soaked into 6X SSC as the blotting buffer. The wet membrane was placed on the top of the gel. All air bubbles trapped between the gel and the membrane were removed. Three sheets of Whatman paper cut at the membrane size and several layer of tissue paper were put on top of the nylon membrane. To ensure a good transfer of the DNA, it was important to prevent any direct contact between the paper-bridge on the gel and the “sucking

paper" on top. Then a glass plate and a weight were placed on the top of the tissue paper. The DNA transfer was allowed to take place overnight. After blotting, the nylon membrane was removed from the gel, soaked in neutralization solution (0.5 M Tris-HCl, 1M NaCl; pH 7.2) for 15 min and allowed to dry. The DNA on nylon membrane was fixed by UV-crosslinking (GS GENE LINKER^{TM} UV chamber, Bio-Rad, USA).

11.4 Preparation of DNA probe for hybridization

The 425-bp *gus* fragment to be used as a probe was obtained from PCR amplification using p3+4 as described in 11.2. The PCR product was precipitated from PCR solution and unwanted salts by the addition of one tenth volume of 3M sodium acetate buffer; pH 5.2 to equalize ion concentrations. After that, at least 2 volumes of cold 100% ethanol were added in the reaction, stored at -20°C for at least 1 h and centrifuged for 15 min at 12,000 rpm 4°C so as to collect the DNA pellet. The supernatant was removed as much as possible with a 1 ml micropipette. The tube was recentrifuged and the rest was removed with a 200 µl pipette. The pellet was washed again with 200 µl of cold 70% ethanol followed by centrifugation at 12,000 rpm 5 min 4°C. The pellet was allowed to air dry at room temperature and resuspended in 20 µl of sterile deionized water. The concentration of DNA probe was quantified with spectrophotometer as described in 13.

The *gus* fragment was radio-labeled using the "random prime DNA labeling kit" from Amersham according to the manufacturer's instruction manual. The method is based on the annealing of a mixture of all possible combinations of small primers to the DNA to be labeled. The complementary strand is then synthesized from the 3'-OH termini of those primers using labeling grade Klenow DNA polymerase enzyme. Addition of a modified radio-labeled deoxynucleoside triphosphate (α^{32}P dNTP) to the reaction results in the incorporation of radioactive nucleotides into the newly synthesized complementary DNA strand. Fifty ng DNA probe was diluted in TE buffer to the 45 µl final volume, heat-denatured by boiling for 5 min at 95-100°C and then left for 5 min at room temperature to allow annealing. The following reagents

were added, 5 µl of 10X reaction buffer, 12 µl of dTTP, dATP and dGTP mix, 2 units of Klenow DNA polymerase enzyme and sterile deionized water to adjust the volume to 45 µl. Then 5 µl of 50 µCi (α^{32}P) d/ctp (specific activity 3,000 Ci/mmol) were added. The reaction mixture was incubated at 37 °C for 20 min. Five µl of 0.2 M EDTA were added to the tube to stop the reaction. This radio-labelled DNA was ready for hybridization step.

11.5 Southern hybridization

Pre-hybridization step

Membrane from Southern blotting were pre-hybridized at 65°C for at least 1 h in a hybridization tube containing 12-18 ml of the hybridization buffer (5X SSC, 20 mM phosphate buffer pH 7.0, 10X Denhard's solution, 7% SDS, 100 µg/ml denatured salmon sperm DNA).

Hybridization step

The radio-labeled probe was denatured either by boiling at 100°C for 5 min and immediately cooled on ice for 5 min, or by incubation with 5 µl of 3 N NaOH at room temperature, for 5 min. Then, the probe was added to the hybridization tube. The hybridization was carried out at 65°C overnight.

Washing step

After the hybridization was completed, the membrane was washed sequentially in the appropriate volume (50 ml) of 3X SSC, 2X SSC, 1X SSC plus 1% SDS at 65°C for 15-30 min each and 0.5X SSC, 0.1X SSC plus 0.5% SDS for 15-20 min each. Then, the membrane was placed between 2 sheets of Saran Wrap plastic film before submitted to autoradiography.

Autoradiography

The washed membrane was placed in contact with an X-ray film (Kodak) in a cassette containing 2 intensifying screens, at -80°C. The exposed film was developed in developer and fixer solutions (Kodak) according to the manufactor's recommendations.

Removal of radioisotope from membrane

Washed membrane was not left completely dried to prevent irreversible binding of the probe. To strip off the probe, the membrane was washed with 200 ml of boiling 0.1% SDS for 2 min and kept wet at 4°C until subsequent use.

12. RNA analysis

12.1 Extraction of total RNA by TRIZOL® Reagent

TRIZOL® Reagent is a complete and ready-to-use reagent for the single-step isolation of total RNA developed by Chomczynski (195). The 50-100 mg of tissue samples were grounded in liquid nitrogen using a mortar and pestle before homogenized in 1 ml of TRIZOL reagent. The homogenized samples were incubated at room temperature for 5 min to permit the complete dissociation of nucleoprotein complexes. After adding of 0.2 ml chloroform, the sample was mixed vigorously and incubated at room temperature for 3 min. The mixture was centrifuged at 12,000 rpm at 4°C for 15 min in order to separate the biphatic mixtures into the phenol-chloroform phase and the colorless-aqueous upperphase. The upperphase was transferred into new tube and mix with 0.5 ml isopropanol to precipitate RNA. The mixture was incubated at room temperature for 10 min and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed and the RNA pellet was washed with 1 ml of cold 70% ethanol before centrifuged at 12,000 rpm for 10 min at 4°C. The washed RNA was air dried and dissolved in RNase-free water. The RNA was quantified by spectroscopic measurement of absorbance at wavelength 260 (A_{260}). RNA was stored at -20°C for routine use while keeping in abosolute ethanol at -80°C was preferred for long-term storage.

12.2 RT-PCR analysis

RT-PCR analysis was performed to quantify the expression of *gus* transgene in transgenic lines using the onestep "Access RT-PCR System" (Promega). This commercial kit provides sensitive, quick and reproducible analysis of even rare RNAs. The system uses AMV Reverse Transcriptase (AMV RT) from Avian Myeloblastosis Virus for first strand DNA synthesis and the thermostable *Tfl*DNA Polymerase from *Thermus flavus* (196) for second strand cDNA synthesis and DNA amplification. The Access RT-PCR System includes an optimized single-buffer system that permits extremely sensitive detection of RNA transcripts without a requirement for buffer additions between the reverse transcription and PCR amplification steps. This simplifies the procedure and reduces the potential for contaminating the samples. In addition, the improved performance of AMV Reverse Transcriptase at elevated temperatures (48°C) in the AMV/ *Tfl*5X reaction buffer minimizes problems encountered with secondary structures in RNA.

Total RNA for RT-PCR was isolated from tissues as described in 12.1. After measured the amount of RNA, total RNA (500 ng) was treated with 1.5 unit of DNase I (Promega) and incubated at 37 °C for 30 min. A 1 µl of stop solution was added to terminate the reaction and incubated at 65 °C for 10 min to inactivate the DNase I. Then the the treated RNA was added to the RT-PCR reaction. The RT-PCR reaction mixture composed of 1X of AMV/*Tfl* buffer, 0.2 mM of dNTP mix (10 mM each dNTP), 1 µM of forward and reverse primers, 1 mM of $MgSO_4$, 0.1 µg/µl of AMV Reverse Transcriptase and *Tfl* DNA polymerase respectively, and nuclease-free water to a final volume of 25 µl per reaction. The reaction mixture was gently mixed, spun down and placed in the DNA thermal cycling. The cycling conditions of RT-PCR included the first strand cDNA synthesis at 48°C for 45 min to activate the reverse transcriptase enzyme and 94°C for 2 min to activate AMV enzyme and to denature RNA/cDNA primer. For the second strand cDNA synthesis and PCR amplification, the conditions were as followed: 40 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 1 min of *gus* fragment or 50°C for 1 min of *actin* fragment, extension at

68°C for 2 min following by extension at 68°C for 7 min. The 10 µl of RT-PCR products was analyzed in 1.5 % agarose gel electrophoresis as described in 4.5.

13. Measurement of nucleic acid concentrations

To quantify the amount of DNA or RNA, the measurement of an absorbance at 260 nm using a spectrophotometer (UV/Vis Spectrophotometer, JENWAY, England) was performed. One OD_{260} corresponds to approximately 50 µg/ml, 40 µg/ml and 20 µg/ml for double-stranded DNA, RNA and oligonucleotides, respectively. The ratio between the absorbance at 260 nm and at 280 nm provides an estimation of the purity of the nucleic acid. The $OD_{260/280}$ ratio of pure DNA or RNA is approximately 1.7-2.0.

14. Determination of promoter methylation using bisulfite genomic sequencing PCR

14.1 Principle

Cytosine methylation within the CaMV 35S promoter was assayed by the technique of bisulfite genomic sequencing (197) with some modifications. The method is based on sodium bisulfite mediated conversion of unmethylated cytosines to uracils in single-strand DNA. The location of some modified bases within a DNA sequence can be determined by digestion with restriction endonucleases which are sensitive to the presence of 5-methylcytosine residues in the specific sequences they recognize, then they are followed by PCR amplification of the resultant modified DNA with primers specific upper or lower strand of interested region. Exact methylation maps of DNA strands from individual genomic DNA molecules can readily be established where the position of each 5-methylcytosine is given a clear positive band on a sequencing gel.

14.2 Procedure

14.2.1 Denaturation

Transgenic plant genomic DNA 10 μg mixed with 90 μg control plant genome DNA were completely digested with appropriate restriction enzyme, which did not cut within the target sequence in which this case was *Hind*III. Then 1 volume of phenol/chloroform were added and mixed, after centrifugation at 10,000 rpm for 10 min at 4°C, the upper phase was transferred into new microcentrifuge tube. The digested DNA was precipitated in 1/10 volume of 3 M sodium acetate and 2 volume of ice-cold absolute ethanol, the mixture was incubated at -20°C for 20 min. After centrifugation at 10,000 rpm for 10 min at 4°C, supernatant was discarded and pellet was resuspended in 127.5 μl deionized water and then chilled on ice. Afterwards, 22.5 μl of 1 N NaOH which was freshly prepared was added and heated denature at 45°C for 10 min. The DNA was quick freezed in dry ice with ethanol and them chilled on ice. A 150 μl of HCl (637.5 μl deionzied water and 112.5 μl 1 N HCl), which was freshly prepared, was added and DNA was precipitated as previously described.

14.2.2 Bisulfite reaction

The DNA pellet was resuspended in 200 μl deionized water and then heated at 95°C for 5 min. After adding 400 μl of freshly prepared bisulfite solution (22.5 M sodium bisulfite, 0.75 mM hydroquinone), the mixture was overlaid with N_2 gas and mineral oil and incubated at 50°C overnight in dark.

The free bisulfite was removed by purification using the Promega wizard DNA clean up system and the final purified DNA solution was 50 μl. The reaction was added with 1/10 volume of freshly prepared of 3 N NaOH and then incubated at 37°C for 30 min. The 6 M of ammonium acetate pH 7 was added. The DNA was precipitated as previously described and washed with 70 % ethanol. The washed DNA pellet was air-dried and dissolved in 200 μl deionized water. The DNA was treated with bisulfite again. Finally, the completely bisulfite-treatment DNA was dissolved in 50 μl deionized water. The concentration of DNA was determined by measuring the optical density at 260 nm.

14.2.3 PCR amplification

The PCR amplification was performed using bisulfite-treated DNA as a template. The reaction was performed in 100 µl reaction mixture. The final concentration of each component was 1X PCR buffer, 1.5 mM $MgCl_2$, 400 µM dNTP mix (100 µM each dNTP), 50 pmol each oligonucleotide (1, 2, 3, 4) primer, 205 units Amplitaq Gold polymerase (Perkin Elmer). The first PCR amplification, 50-100 ng of bisulfite-treated DNA, was used as a template and 2 µl of PCR product from the first PCR amplification was used as a template in the second or nested PCR amplification. In the first PCR amplification, U1-EX34 and U2-EX32 primers were used for upper strand amplification, and L1-EX32 and L2-EX33 primers were used for lower strand amplification (table 7). Then in the second PCR, U1-IN32 and U2-IN32 primers were used for upper strand amplification, and L1-IN32 and L2-IN26 were used for lower strand amplification (table 7). The reaction mixture was gently mixed and spun down. The PCR program consisted in one cycle at 95°C for 2 min 30 s to completely melt the two-strand of genomic DNA, then 32 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min for the first PCR amplification and 60°C for the second round of PCR and extension at 72 °C for 1 min. After the last cycle, extension at 72°C for 10 min was performed to fulfill polymerization. A second round of PCR was performed with 5 µl of the reaction from the first round. The PCR products were stored at 4°C.

Table 7. Sequences of the primers used for PCR amplification of bisulfite-treated genome DNA coding for CaMV 35S promoter region.

sequence	PCR	upper strand		lower strand	
		primer (5' → 3')	Tm (°C)	primer (5' → 3')	Tm (°C)
CaMV 35S pro	first	U1-EX34: CCTCTA(A/G)A(A/G)TCC CC(A/G)T(A/G)TTCTCTCCAAAT(A/ G)AAAT U2-EX32: GT(C/T)A(C/T)TTTATTGT GAAGATAGTGGAAAAGGAA	55	L1-EX32: CC(A/G)(A/G)AAACC T(A/G)(A/G)TC(A/G)(A/G)ATTCCATA (A/G)T(A/G)(A/G)AAAA L2-EX33: GAT(C/T)T(C/T)AGGG GG(C/T)A(C/T)AAGAGAGGTTT TA(C/T)TTTA	55
	second	U1-IN32: TTCTCTCCAAAT(AG)AA AT(A/G)AACTTCCTTATATA U2-IN32: ATAGTGGAAAAGGAAG GTGG(C/T)T(C/T)(C/T)TA(C/T)AAAT G	60	L1-IN32: ATA(A/G)T(A/G)(A/G)A AAA(A/G)(A/G)AA(A/G)(A/G)T (A/G)(A/G)CTCCTAC L2-IN26: AGGTTTA(C/T)TTTA (C/T)TTGAAGGAATATA	60

CHAPTER 4
RESULTS

PART I Study of gene transformation in tobacco

1. *Agrobacterium*-mediated transformation

1.1 Establishment of transformation system

The *Agrobacterium* strain EHA105 and AGL1, which harbor the plasmid pCAMBIA1301, were used for leaf disk transformation. Leaf disks of tobacco plants, *Nicotiana tabacum* cv. Samson NN, were cut into small pieces (0.8×0.8 cm), immersed in *Agrobacterium* suspension and co-cultivated for 2 days to allow gene transformation. After cultured on selective medium, RMOP containing 50 mg/l hygromycin B and 250 mg/l carbenicillin, we found that *Agrobacterium* strain EHA105 gave higher transformation efficiency as compared to the strain AGL1. Therefore, the strain EHA105 was employed. The 18% of resistant calli were recovered within 3-4 weeks (Fig. 20A). Resistant shoots were regenerated from the calli around 4-5 weeks later (Fig. 20B) with an average of 6 shoots/callus while control explants plated on nonselective medium showed 10-15 shoots/callus. The regenerated shoots were separated from resistant calli and transferred to MS medium containing 50 mg/l hygromycin B and 250 mg/l carbenicillin for root formation (Fig. 20C). Tobacco plants that contaminated with *Agrobacterium* or lacked of roots were discarded. To maintain the transgenic lines, 4-week-old putative transgenic plants were micropropagated on MS medium containing 50 mg/l hygromycin B by sequential *in vitro* subculture from apical bud or auxiliary bud segments.

Transient expression of *gus* gene was analyzed in leaf disks after 2 days of co-cultivation. Tobacco leaves were incubated in X-Gluc solution at 37°C overnight. Blue spots were observed after chlorophyll extraction by shaking for several hours in

70% alcohol. The presence of blue spots in a transformed leaf was shown in Fig. 21A. GUS assay was done in regenerated shoots as shown in Fig. 21B. Stable expression of *gus* transgene was also verified in 13 lines of the transgenic plants. The results from blue staining revealed that all of transgenic leaves maintained GUS activities, which could be classified into 2 groups, moderate GUS-expressing plants and intensive GUS- expressing plants, as shown in Fig. 21C and 21D.

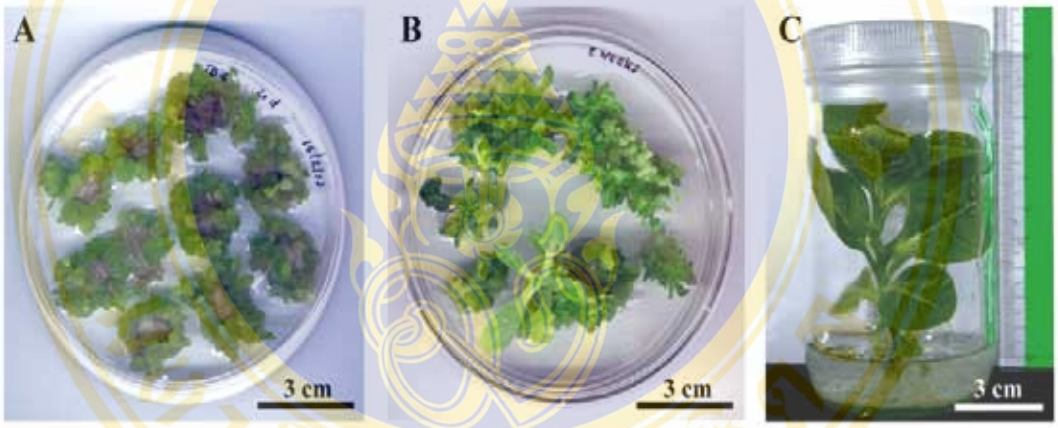

Figure 20. Regeneration of transgenic tobacco plants using *Agrobacterium*-mediated transformation

(A) Formation of resistant calli after cultured on RMOP medium containing 50 mg/l hygromycin B for 3 weeks, (B) Regeneration of resistant shoots on RMOP medium containing 50 mg/l hygromycin B after cultured for 8 weeks, (C) The 2-month-old transgenic plant cultured on MS supplemented with 50 mg/l hygromycin B.

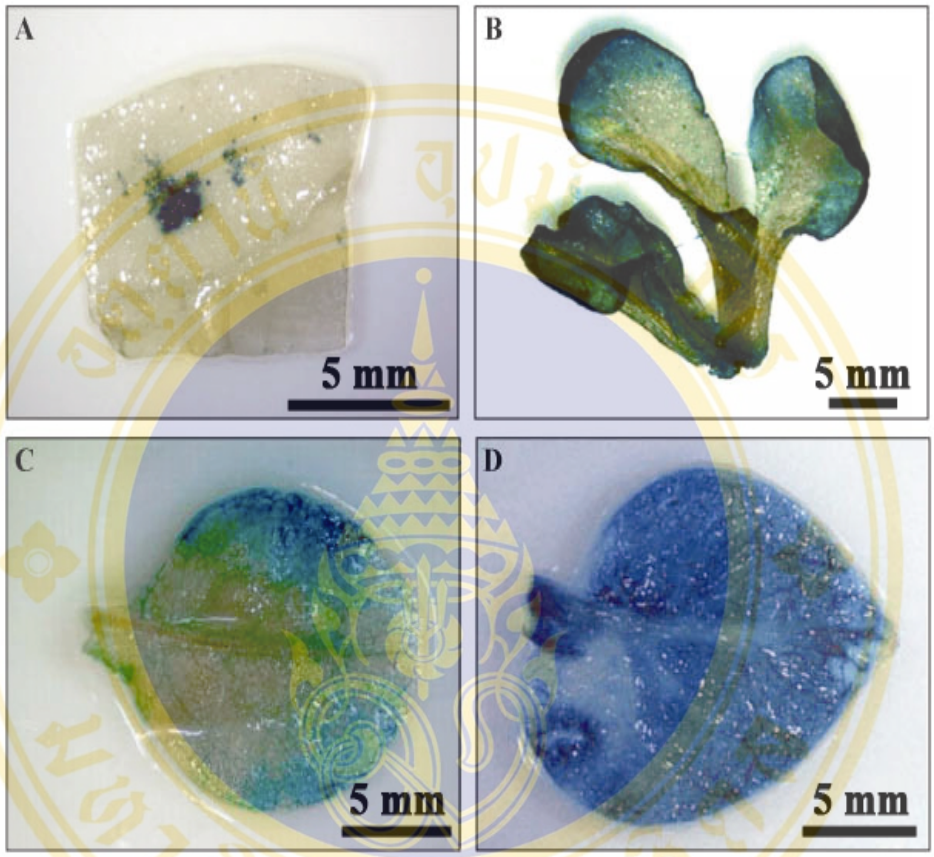

Figure 21. Histochemical analysis of transgenic tobacco from *Agrobacterium*-mediated transformation

(A) Transient expression at 2 days after co-cultivation, (B) GUS expression in regenerated shoot, (C, D) Differential expression of *gus* gene, (C) moderate GUS-expressing plant (line 4.5) and (D) intensive GUS-expressing plant (line 4.10)

1.2 Molecular characterization and transgene integration analysis

1.2.1 PCR analysis

PCR amplifications of 4 regions of *gus* (p3+4), *hpt* (p5+6), CaMV 35S-*gus* (p7+9) and CaMV 35S-*hpt* (p7+8) were examined for the presence of transgenes in 13 transgenic lines. As a result, all of them showed the existence of *gus* gene and *hpt* gene with complete regions of CaMV 35S promoter (Fig. 22-25). Rearrangement of T-DNA integration was also investigated using p1+2, p1+1 and p2+2 primers for direct repeat (head to tail) and inverted repeats (head to head and tail to tail) formation, respectively, as illustrated in Fig. 19. PCR products could not be amplified by these 3 primer pairs, thus we concluded that there was no rearrangement of T-DNA in these transgenic tobacco obtained from *Agrobacterium*-mediated transformation.

1.2.2 Southern hybridization

To produce *gus* probe for Southern hybridization, the 425-bp *gus* fragment from pCAMBIA1301 was amplified by primer 3+4. The PCR product was purified and labeled with ^{32}P to be used as hybridization probe. Genomic DNA was extracted from transgenic leaves as described in materials and methods and digested with *Hin*dIII. Since the T-DNA region of the pCAMBIA1301 has only one *Hin*dIII site, which is present in between the *hpt* and the *gus* genes (Fig. 19A), digestion of the genomic DNA of transgenic plants with *Hin*dIII generates a unique fragment for each integrated copy. Therefore, the Southern blot results may provide an estimate of the copy number of the *gus* gene in the genome of the transgenic plants. The results revealed that the number of *gus* transgene in both moderate (A4.5) and intensive (A4.10) GUS-expressing plants was only 1 copy (Fig. 26). This investigation indicated a simple pattern of transgene integration and stability of *gus* transgene with no rearrangement in both moderate and intensive GUS-expressing lines. Hence, the differential expressions of *gus* gene were not due to the copy number of an introduced transgene. It should be noted that the integration site of the transgene on genome of the moderate (A4.5) and intensive (A4.10) GUS-expressing lines were different as seen by different sizes of transgene integrated fragment generated by *Hin*dIII.

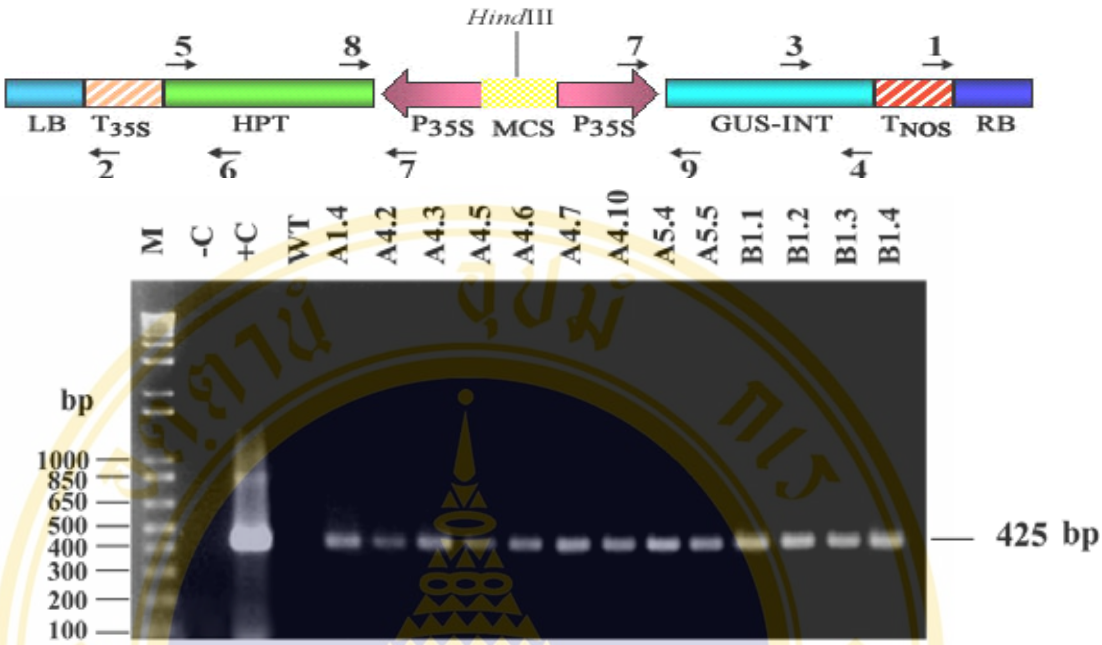

Figure 22. PCR amplification of *gus* gene in putative transgenic tobacco plants obtained from *Agrobacterium*-mediated transformation using primer 3+4; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: plasmid pCAMBIA1301 as a positive control, WT: wild type tobacco. Gel electrophoresis was performed in 1.5% (w/v) agarose.

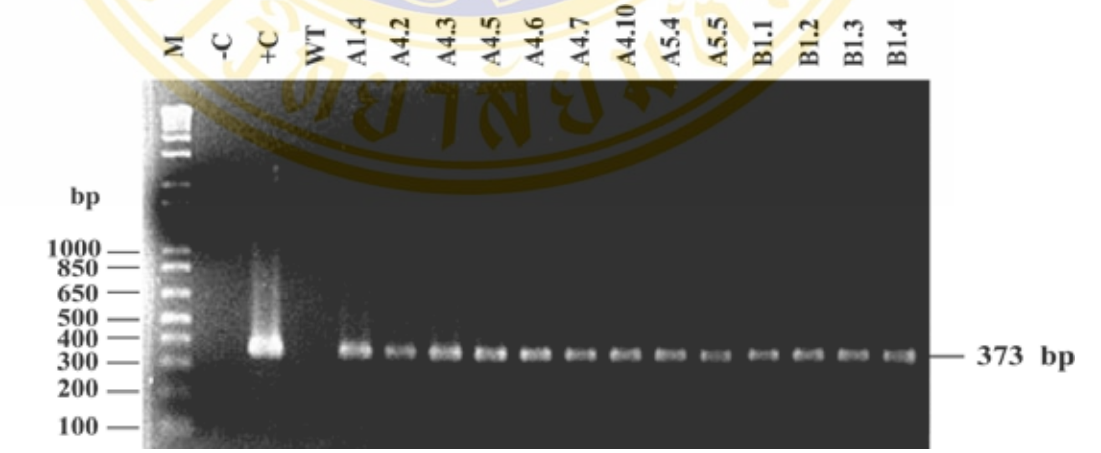

Figure 23. PCR amplification of *hpt* gene in putative transgenic tobacco plants obatained from *Agrobacterium*-mediated transformation using primer 5+6; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: plasmid pCAMBIA1301 as a positive control, WT: wild type tobacco. Gel electrophoresis was performed in 1.5% (w/v) agarose.

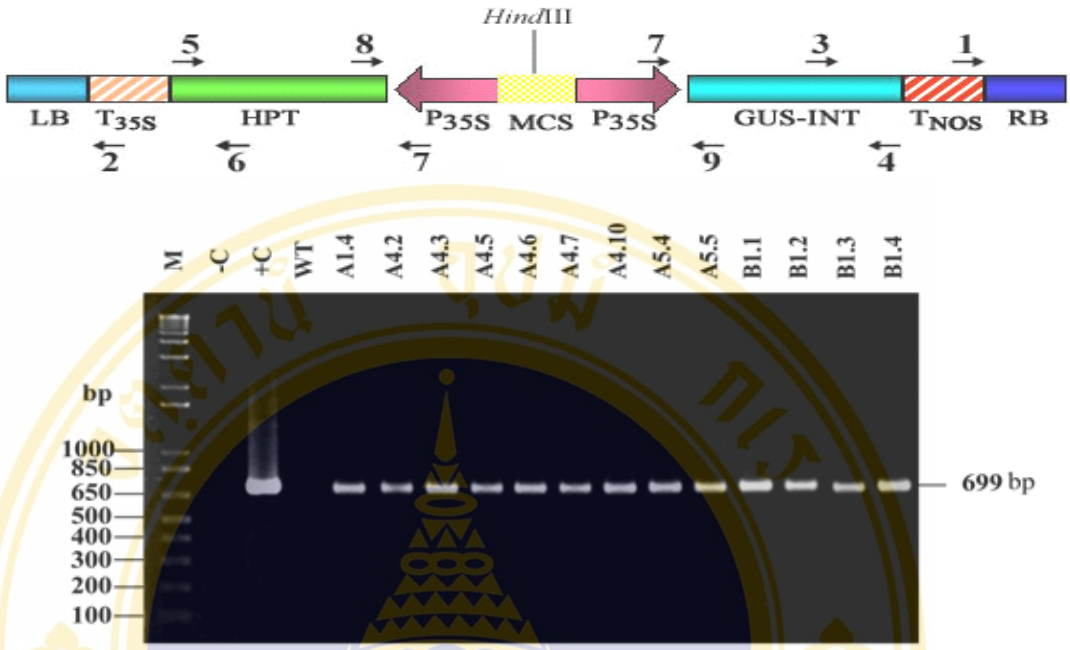

Figure 24. PCR amplification of CaMV 35S-*gus* region in putative transgenic tobacco plants obtained from *Agrobacterium*-mediated transformation using primer 7+9; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: plasmid pCAMBIA1301 as a positive control, WT: wild type tobacco. Gel electrophoresis was performed in 1.5% (w/v) agarose.

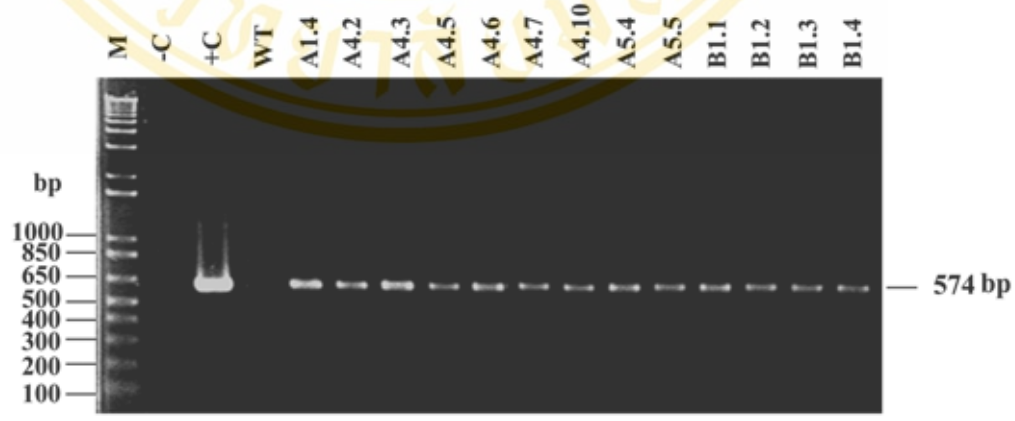

Figure 25. PCR amplification of CaMV 35S-*hpt* region in putative transgenic tobacco plants obtained from *Agrobacterium*-mediated transformation using primer 7+8; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: plasmid pCAMBIA1301 as a positive control, WT: wild type tobacco. Gel electrophoresis was performed in 1.5% (w/v) agarose.

Figure 26. Southern hybridization of *gus* gene in differential GUS expressing lines of transgenic tobacco obtained from *Agrobacterium*-mediated transformation; the 425-bp fragment of *gus* gene was labeled with ^{32}P and used as a probe.

1.3 Determination of *gus* expression by RT-PCR

To determine the level of transcription in transgenic tobacco, RT-PCR analysis method was performed. Total RNA of transgenic tobacco was extracted from the moderate GUS-expressing plant (line A4.5) and intensive GUS-expressing plant (line A4.10). RT-PCR analysis of *gus* transgene was determined using primer 3+4 as described in materials and methods. The results implied that, transcription of *gus* gene was gradually increased from the moderate GUS-expressing plant to the intensive one as shown in Fig. 27.

Figure 27. RT-PCR analysis of RNA transcribed from differential GUS expressing tobacco obtained from *Agrobacterium*-mediated transformation in 1.5% (w/v) agarose gel electrophoresis; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: pCAMBIA1301 as a positive control, WT: wild type plant. The *actin* gene expression was determined as a control of housekeeping gene.

1.4 DNA methylation status analysis

From RT-PCR analysis, we concluded that silencing of *gus* transgene occurred at the transcription level (TGS). Therefore, we also analyzed DNA methylation at promoter region of transgene in order to investigate the correlation of DNA methylation and transcriptional gene silencing. In this study, we evaluated the methylation status in CaMV 35S promoter in moderate (A4.5) and intensive (A4.10) GUS-expressing tobacco plants using bisulfite genomic sequencing PCR technique. Genomic DNA of transgenic line A4.5 and A4.10 was treated with bisulfite that converted the unmethylated cytosine into uracil, which finally appeared as thymidine in PCR product, whereas all ^{m}C residues remain unaltered. After genomic sequencing, the percentage of ^{m}C to total cytosine residues (%^{m}C/total C) as well as the percentage of ^{m}C to total specific sequences of asymmetrical and symmetrical (%^{m}C/No.) residues, in specific sequences of CaMV 35S promoter region were calculated. Asymetrical sequences were CA, CT, CC while symmetric sequences were CG and CNG (N=A, T, C or G). Analyses of ^{m}C distribution in upper and lower strands of CaMV 35S promoter region were shown in Table 8 and Fig. 28. The methylation event at CaMV 35S promoter showed the association with degree of *gus* expression. Level of cytosine methylation was highly detected in the moderate GUS-expressing tobacco rather than the intensive GUS-expressing tobacco. In the intensive GUS-expressing plant (A4.10), C residues located within the symmetrical sequences; CG and CNG, of upper and lower strands were not methylated whereas C residues in asymmetrical sequences; CC, CT and CA, were slightly methylated in lower strand. On the contrary, methylations of cytosines in the moderate GUS-expressing plant (A4.5) were occurred in both symmetrical and asymmetrical sequences with highest level in CG region.

Table 8. Percentage of methylated cytosine to total cytosine and to total specific sequences of upper and lower strands of CaMV 35S promoter region in transgenic tobacco

A: %^{m}C/total C was percentage of methylated cytosine to total cytosine residues in specific CaMV 35S region. Asymmetrical sequence were CA, CT, CC and symmetrical sequence were CG, CNG (N=A, T, C, G).

B: % ^{m}C/No. was percentage of methylated cytosine to total specific sequence CA, CT, CC, CG and CNG in CaMV 35S region.

(A)

%^{m}C/Total C	Upper strand		Lower strand	
	A4.10	**A4.5**	**A4.10**	**A4.5**
CA	0	0	4	7
CT	8	11	7	9
CC	0	19	7	7
Total asym. Seq	**8**	**30**	**18**	**23**
CG	0	16	0	20
CNG	0	5	0	4
Total sym. Seq	**0**	**21**	**0**	**24**

(B)

%^{m}C/No.	Upper strand		Lower strand	
	A4.10	**A4.5**	**A4.10**	**A4.5**
CA	0	0	18	27
CT	20	27	25	34
CC	0	55	33	33
CG	0	90	0	90
CNG	0	66	0	67

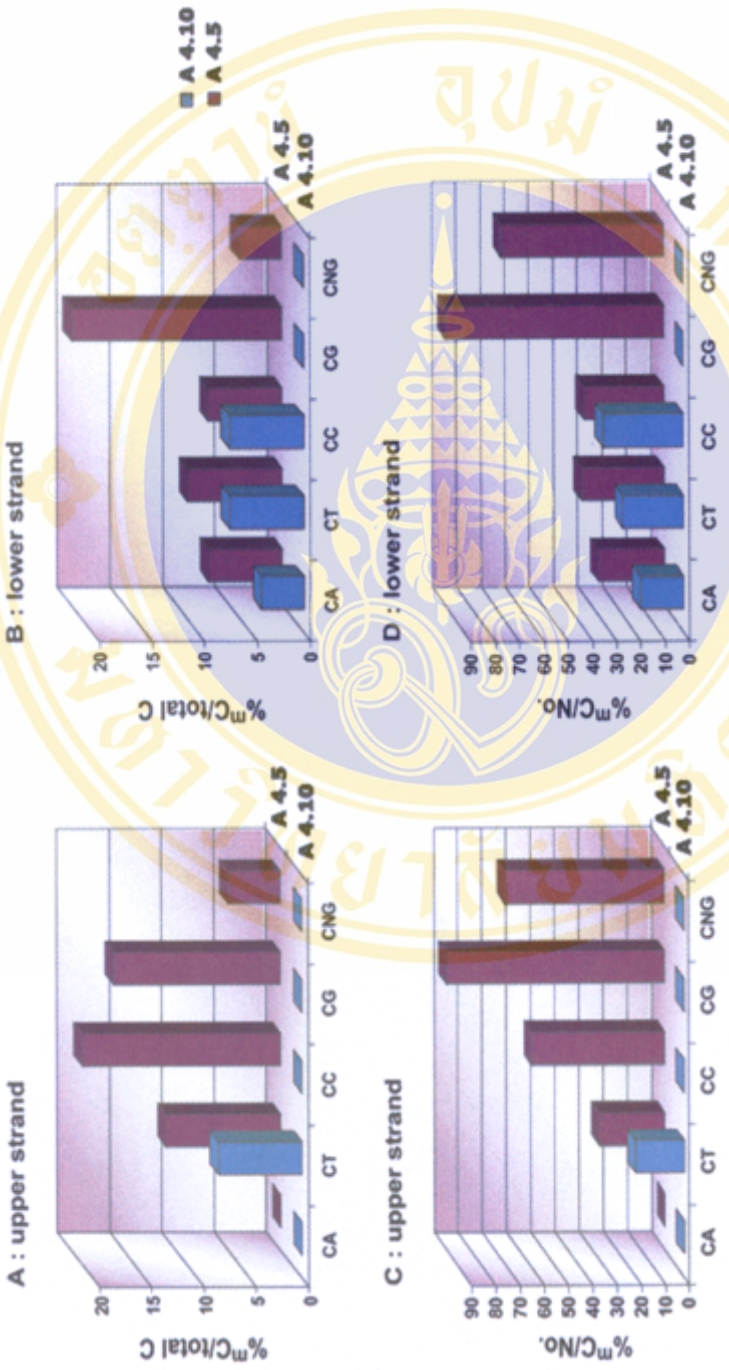

Figure 28. Diagrams of the percentage of methylated cytosine to total cytosine and to total specific sequences of upper and lower strands of CaMV 35S promoter in differential GUS expressing tobacco plants obtained from *Agrobacterium*-mediated transformation; A4.10 and A4.5 were highly and moderate GUS expressing plants, respectively. A, B: %^{m}C/total C was percentage of methylated cytosine to total cytosine residues in CaMV 35S promoter region. C, D: %^{m}C/No. was percentage of methylated cytosine to total specific sequence CA, CT, CC, CG, CNG in CaMV 35S promoter region.

2. Particle bombardment

2.1 Establishment of transformation system

To perform the bombardment, tobacco leaves were placed abaxial side up on top of a sterile filter disk at the center of the plate on an RMOP medium. The cut leaves were precultured overnight before bombardment. A bombardment was carried out using the biolistic PDS-1000/He (Bio-Rad) Particle Delivery system. Plasmid pCAMBIA1301 was prepared and precipitated onto 1.0 μm gold particles as described in materials and methods. The bombardment chamber was evacuated at a pressure of 28 inches of mercury. All bombardments were conducted with a pulse of 1,100 psi at a range of 9 cm target distance. After bombardment, the leaves were transferred to fresh RMOP medium and cultured at 26°C under a 16-h photoperiod for 2 days. The bombarded leaves were cut into small pieces (0.8×0.8 cm) before transferred to RMOP medium containing 50 mg/l hygromycin B. Some of the bombarded pieces were subjected to histochemical assay to observe the transient expression. In consequence, a few blue spots were observed in bombarded tissues (data not shown); however, some of them were further cultured on the selective medium, RMOP containing 50 mg/l hygromycin B. Resistant calli were observed after cultured for 3-4 weeks with an average of 7%. Tobacco shoots were regenerated 5 weeks later (Fig. 29B) with the average of 1.42±0.53 shoots/resistant callus, whereas average regeneration frequency in non-transformed explants was 8.54±3.05 shoots/resistant callus. The putative transgenic shoots were transferred to fresh MS medium supplemented with 50 mg/l hygromycin B so as to get the whole plants. Four-week-old transgenic leaves from 10 tobacco plants were assayed for GUS activities. The outcomes revealed that only 30% of them gave positive results (Table 9, Fig. 29A). All of them were further analyzed in molecular analysis.

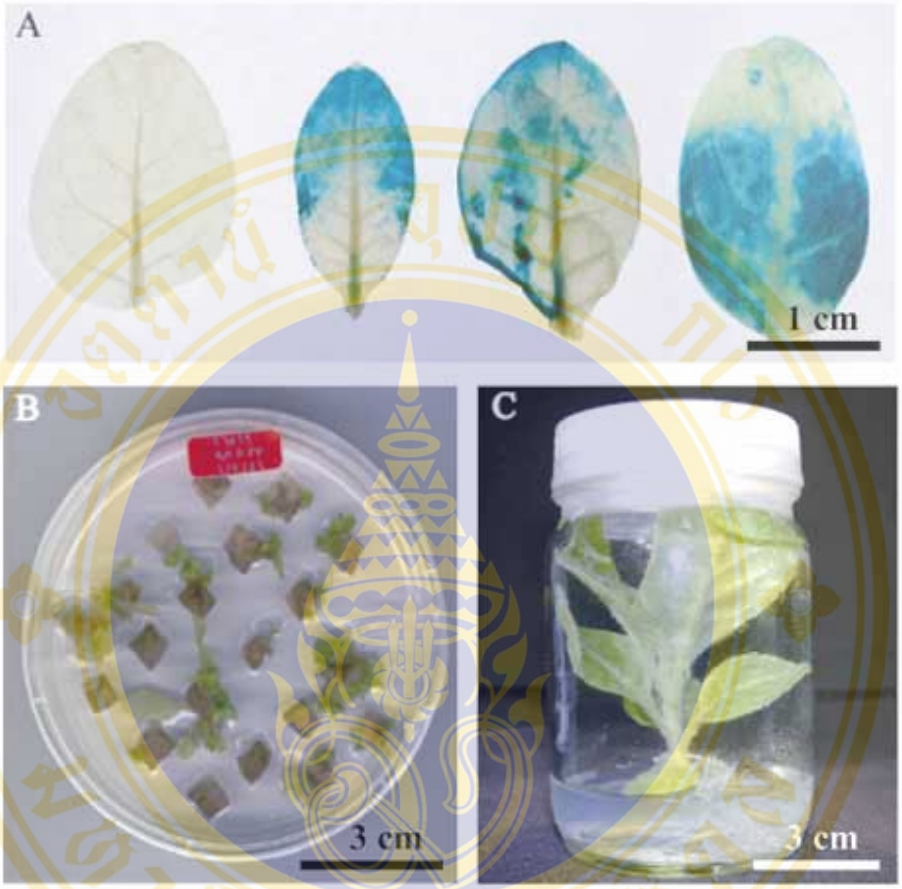

Figure 29. Particle bombardment in tobacco

(A) Differential expression of GUS in regenerated plants, (B) Six-weeks resistant shoots regenerated on RMOP + 50 mg/l hygromycin B, (C) Transgenic plant growing on MS medium + 50 mg/l hyromycin B

Table 9. The number of GUS-expressing plants in 10 regenerated tobacco plants from 7 hygromycin-resistant callus lines obtained from particle bombardment

Resistant line	Number of GUS-expressing plants/regenerated plants
1	0/2 (0%)
2	0/2 (0%)
3	1/2 (50%)
4	1/1 (100%)
5	1/1 (100%)
6	0/1 (0%)
7	0/1 (0%)
Total	**3/10 (30%)**

2.2 Molecular characterization and transgene integration analysis

2.2.1 PCR analysis

The presence of transgenes and integration events were examined in 10 lines of putative transgenic tobacco by PCR amplification with 4 primer pairs: 3+4 (*gus*), 5+6 (*hpt*), 7+9 (CaMV 35S-*gus*) and 7+8 (CaMV 35S-*hpt*), as a result, the absence of *gus* gene and CaMV 35S-*gus* fragments were observed in 7 out of 10 lines as reported in Fig. 30-33 and Table 10. This investigation revealed that the silence of *gus* transgene, which was detected by histochemical analysis, resulted from transgene integration event. None of this evidence was observed in *hpt* gene because transgenic plants were cultured on hygromycin-contained medium so the plant that lacked of *hpt* gene could not recover under this stress condition. We also investigated the rearrangement of T-DNA (Fig. 19) with primer 1+1, 1+2 and 2+2 in the complete transgene integration lines (BA3.2, BA4.1 and BA5.1). Nevertheless, transgene rearrangement patterns could not be detected in those transgenic plants.

Table 10. Different patterns of transgene integration in transgenic tobacco plants obtained from particle bombardment

Line	Histochemical analysis	PCR amplification			
		gus	35S-*gus*	*hpt*	35S-*hpt*
BA1.1	-	-	-	+	+
BA1.2	-	-	-	+	+
BA2.1	-	-	-	+	+
BA2.2	-	-	-	+	+
BA3.1	-	-	-	+	+
BA3.2	+	+	+	+	+
BA4.1	+	+	+	+	+
BA5.1	+	+	+	+	+
BA6.1	-	-	-	+	+
BA7.1	-	-	-	+	+

- = PCR negative

+ = PCR positive

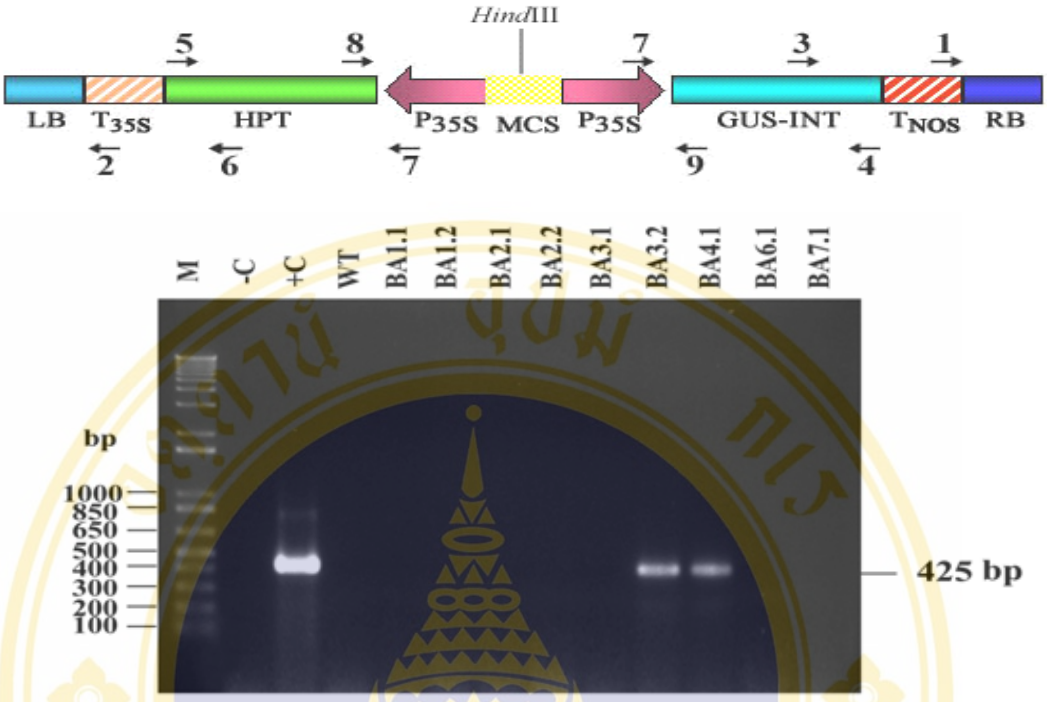

Figure 30. PCR amplification of *gus* gene in putative transgenic tobacco plants obatained from particle bombardment using primers 3+4; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: plasmid pCAMBIA1301 as a positive control, WT: wild type tobacco. Gel electrophoresis was performed in 1.5% (w/v) agarose.

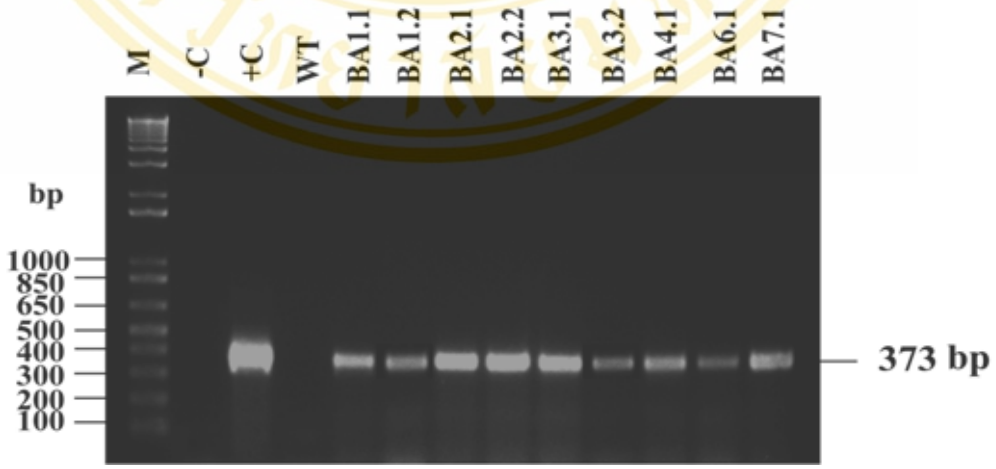

Figure 31. PCR amplification of *hpt* gene in putative transgenic tobacco plants obatained from particle bombardment using primers 5+6; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: plasmid pCAMBIA1301 as a positive control, WT: wild type tobacco. Gel electrophoresis was performed in 1.5% (w/v) agarose.

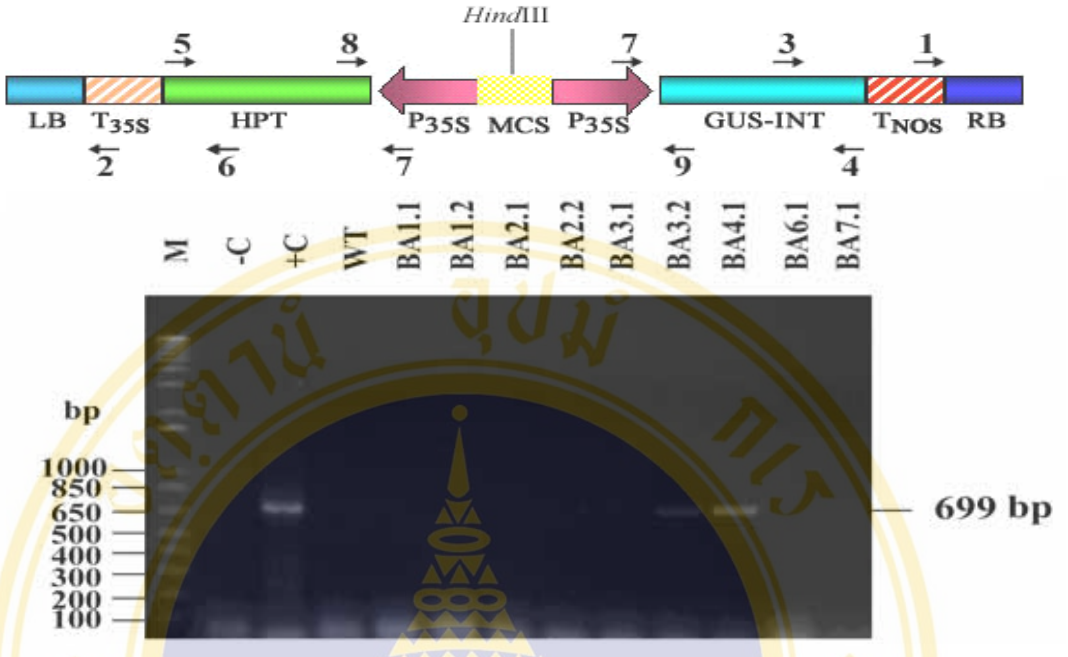

Figure 32. PCR amplification of CaMV 35S-*gus* region in putative transgenic tobacco plants obtained from particle bombardment using primer 7+9; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: plasmid pCAMBIA1301 as a positive control, WT: wild type tobacco. Gel electrophoresis was performed in 1.5% (w/v) agarose.

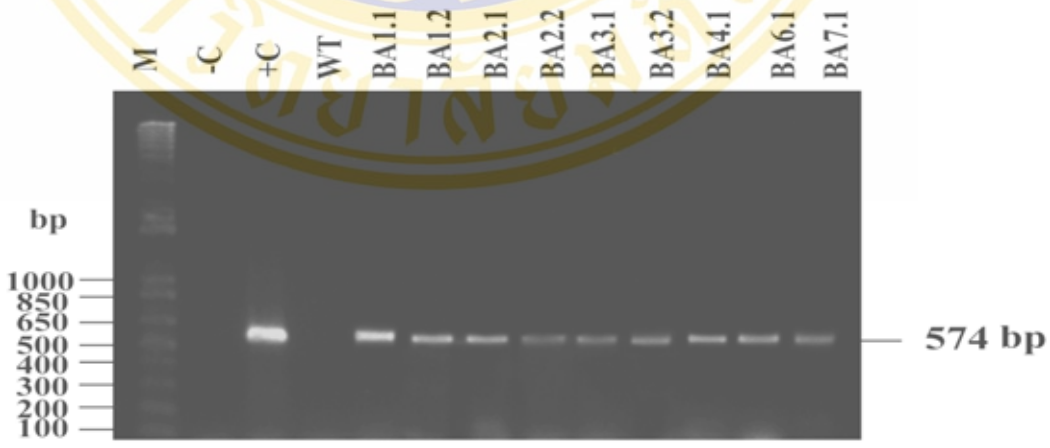

Figure 33. PCR amplification of CaMV 35S-*hpt* region in putative transgenic tobacco plants obtained from particle bombardment using primer 7+8; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: plasmid pCAMBIA1301 as a positive control, WT: wild type tobacco. Gel electrophoresis was performed in 1.5% (w/v) agarose.

PART II Study of gene transformation in *japonica* rice

1 *Agrobacterium*-mediated transformation

Rice calli (*Oryza sativa* spp. *japonica* cv. Taichung) were transformed by *Agrobacterium*-mediated transformation using bacterial strain EHA105, which carries the plasmid pCAMBIA1301 encoding an intron-containing β-glucuronidase (*gus-int*) gene and a hygromycin phosphotransferase (*hpt*) gene. Three weeks after co-cultivation, resistant calli were observed on the selective medium, N6D supplemented with 50 mg/l hygromycin B and 250 mg/l carbenicillin (Fig 34A). The resistant calli were then transferred to regeneration medium containing the same concentration of antibiotics for a period of 3-4weeks. Plantlets were regenerated (Fig. 34B) and, subsequently, shoots began to roots when transferred to root induction medium (Fig. 34C). The frequency of regenerated plants/resistant callus was 5±2.19 while control explants plated on nonselective medium showed the average regenerated frequency of 7±1.57 regenerated plants/resistant callus. GUS histochemical assays were analyzed in both transformed calli 3 days after transformation (Fig 35A, B) and leaves of 30 putative transgenic plants regenerated from 6 callus lines. 43% of the plants had GUS expression (Table 11). Moreover, we also characterized the differential patterns of GUS staining into three levels: silent, moderate and intensive GUS-expressing plants as shown in Fig. 35C, D and E, respectively.

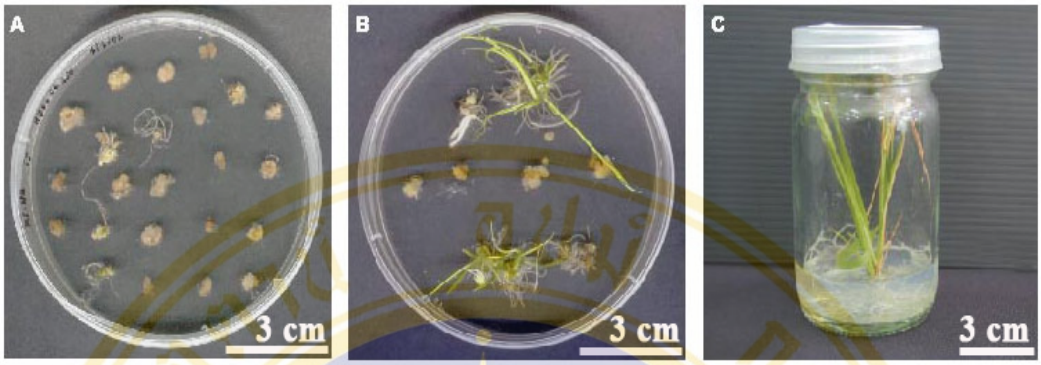

Figure 34. Regeneration of transgenic *japonica* rice

(A) 3-weeks hygromycin-resistant calli on N6D containing 250 mg/l carbenicillin and 50 mg/l hygromycin B, (B) Regeneration of transgenic rice from the resistant calli after cultured on MS-NK medium for 4 weeks, (C) Transgenic rice plant on MS-HF medium.

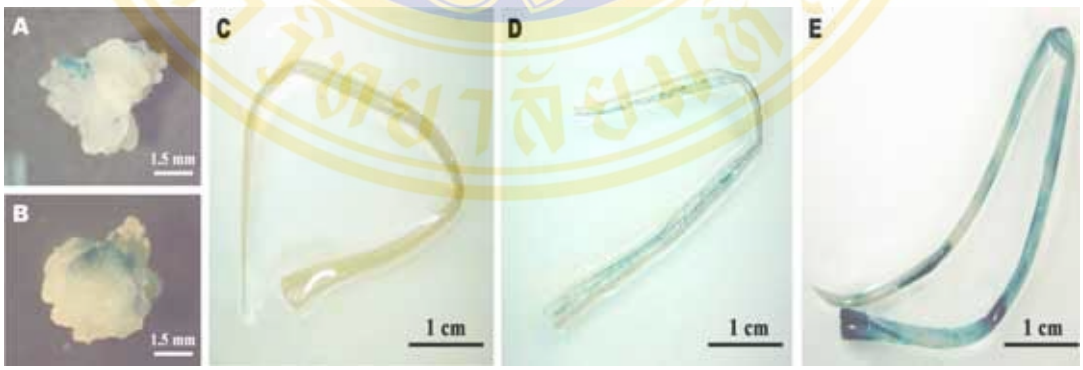

Figure 35. Histochemical analysis of transgenic *japonica* rice

(A-B) Transient expression in callus, 3 days after co-cultivation, (C-E) Differential expression of *gus* gene in transgenic leaves: (C) Silent plant (line 2.2), (D) Moderate GUS expressing plant (line 6.3), (E) Intensive GUS expressing plant (line 4.5).

Table 11. The number of GUS-expressing plants in 30 regenerated *japonica* rice from 6 hygromycin-resistant callus lines obtained from *Agrobacterium*-mediated transformation

Resistant line	Number of GUS-expressing plants/regenerated plants
1	0/6 (0%)
2	0/6 (0%)
3	3/7 (42%)
4	5/6 (83%)
5	1/1 (100%)
6	4/4 (100%)
Total	**13/30 (43%)**

2. Molecular characterization and transgene integration analysis

2.1 PCR analysis

Genomic DNA of 30 putative transgenic *japonica* (cv. Taichung) was extracted from rice leaves. Amplifications by polymerase chain reaction were examined with 4 primer pairs (*gus, hpt,* CaMV 35S-*gus* and CaMV 35S-*hpt*) for the determination of transgene integrations as well as the study in transgenic tobacco as mentioned above. The results showed that, *hpt* gene could be amplified in every regenerated plant while the absence of *gus* fragment was observed in line J1.1, J1.2, J1.3, J1.5, J3.1, J3.2 and J3.3. In addition, the region of CaMV 35S-*gus* was undetectable in line J1.1-1.6, J2.3-2.6, J3.1-3.3, J3.7 and J4.1, so we could not observed GUS activities in these transgenic lines by histochemical analysis. (Table 12). To detect the rearrangement of T-DNA, amplifications with p1+1, p1+2 and p2+2 were performed in all transgenic lines; however, there were no rearrangement events observed.

Table 12. Different patterns of transgene integration in transgenic *japonica* rice

Line	Histochemical analysis	PCR amplification			
		gus	35S-*gus*	*hpt*	35S-*hpt*
J1.1	-	-	-	+	+
J1.2	-	-	-	+	+
J1.3	-	-	-	+	+
J1.4	-	+	-	+	+
J1.5	-	-	-	+	+
J1.6	-	+	-	+	+
J2.1	-	+	+	+	+
J2.2	-	+	+	+	+
J2.3	-	+	-	+	+
J2.4	-	+	-	+	+
J2.5	-	+	-	+	+
J2.6	-	+	-	+	+
J3.1	-	-	-	+	+
J3.2	-	-	-	+	+
J3.3	-	-	-	+	+
J3.4	+	+	+	+	+
J3.5	+	+	+	+	+
J3.6	+	+	+	+	+
J3.7	-	+	-	+	+
J4.1	-	+	-	+	+
J4.2	+	+	+	+	+
J4.3	+	+	+	+	+
J4.4	+	+	+	+	+
J4.5	+	+	+	+	+
J4.6	+	+	+	+	+
J5.1	+	+	+	+	+
J6.1	+	+	+	+	+
J6.2	+	+	+	+	+
J6.3	+	+	+	+	+
J6.4	+	+	+	+	+

- = PCR negative

+ = PCR positive

2.2 Southern hybridization

In order to study the correlation between differential expressions and copy number of *gus* gene in transgenic rice, southern hybridization was performed in the candidates of silent (J2.2), moderate (J6.3) and intensive (J4.5) GUS expressing plants. The radiolabeled 425-bp *gus* fragment was hybridized with genomic DNA which was digested with *Hin*dIII as same as the study in transgenic tobacco. Consequently, we detected a single copy of *gus* gene in the silent GUS-expressing line and moderate GUS-expressing line while 2 copies were presented in the intensive one. The results were shown in Fig. 36.

Figure 36. Southern hybridization of *gus* gene in differential GUS expressing lines of transgenic *japonica*; the 425 bp fragment of *gus* was labeled with ^{32}P to be used as a probe.

3. Determination of *gus* expression by RT-PCR

RT-PCR analysis was evaluated in transgenic *japonica* rice that showed different patterns of GUS staining (line J2.2 for silent expression, line J6.3 for moderate and line J4.5 for intensive expression) so as to determine the levels of *gus* transcription. The results revealed that low amount of *gus* transcript respected to gene silencing in line J2.2. The transcripts increased relatively from the silent line to the GUS-expressing lines as shown in Fig. 37.

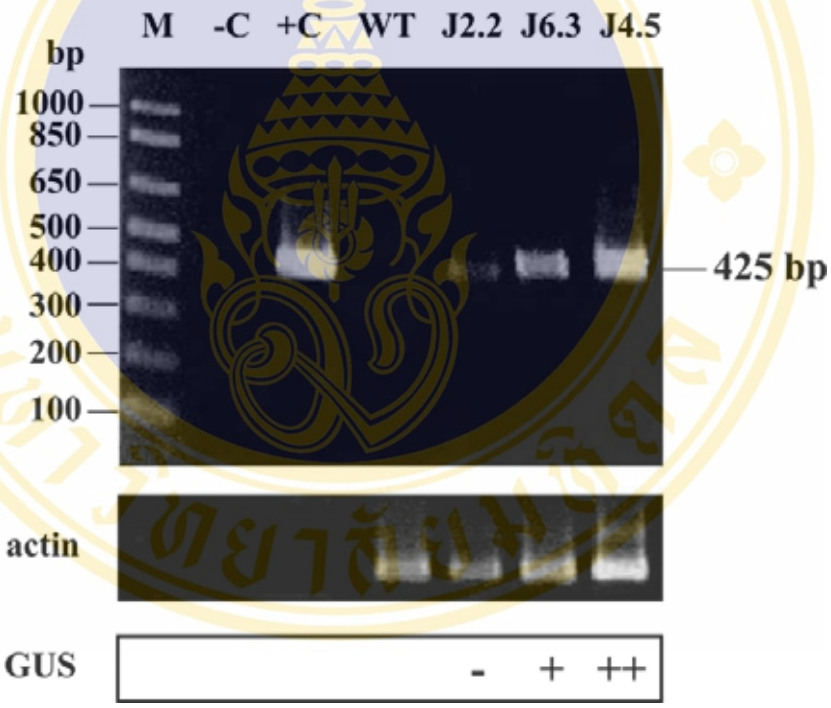

Figure 37. RT-PCR analysis of RNA transcribed from differential GUS expressing *japonica* in 1.5% (w/v) agarose gel electrophoresis; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: pCAMBIA1301 as a positive control, WT: wild type plant. The *actin* gene expression was determined as a control of housekeeping gene.

4. DNA methylation status analysis

The results of genomic sequencing indicated the presence of methylated cytosines at CaMV 35S promoter region in transgenic *japonica* rice obtained from *Agrobacterium*-mediated transformation. Genomic DNA of transgenic line J2.2, J6.3 and J4.5, represented as silent, moderate and intensive GUS expressing plants, were treated with sodium bisulfite and subjected to PCR amplification as described above. The specific primers were designed to be able to amplify specific regions of upper or lower strand of CaMV 35S promoter. After that the 207-bp PCR product was sequenced in order to identify the number of methylated cytosines. Analyses of ^{m}C distribution in upper and lower strands of CaMV 35S promoter regions were shown in Table 13 and Fig. 38. High level of ^{m}C was occurred in the silent plant (J2.2) rather than the moderate GUS- expressing plant (J6.3) and the intensive GUS-expressing plant (J4.5), respectively. In this promoter, methylation of C at CG and CNG to total specific sequence CG and CNG (%^{m}C/No.) were 100% in line J2.2 in both upper and lower strands. Moreover, the methylation was frequently occurred at CA and CC but not CT.

Table 13. Percentage of methylated cytosine to total cytosine and to total specific sequences of upper and lower strands of CaMV 35S promoter region in transgenic *japonica* rice

A: %^{m}C/total C was percentage of methylated cytosine to total cytosine residues in specific CaMV 35S region. Asymmetrical sequence were CA, CT, CC and symmetrical sequence were CG, CNG (N=A, T, C, G).

B: % ^{m}C/No. was percentage of methylated cytosine to total specific sequence CA, CT, CC, CG and CNG in CaMV 35S region.

(A)

%^{m}C/Total C	**Upper strand**			**Lower strand**		
	J4.5	**J6.3**	**J2.2**	**J4.5**	**J6.3**	**J2.2**
CA	0	0	3	0	4	7
CT	8	8	23	11	7	9
CC	0	14	17	19	7	7
Total asym. Seq	**8**	**22**	**43**	**30**	**18**	**23**
CG	0	14	0	16	0	20
CNG	0	3	0	5	0	4
Total sym. Seq	**0**	**17**	**0**	**21**	**0**	**24**

(B)

%^{m}C/No.	**Upper strand**			**Lower strand**		
	J4.5	**J6.3**	**J2.2**	**J4.5**	**J6.3**	**J2.2**
CA	0	0	81	17	27	67
CT	20	27	24	24	34	36
CC	0	55	65	35	33	79
CG	0	89	100	0	91	100
CNG	0	68	100	0	66	100

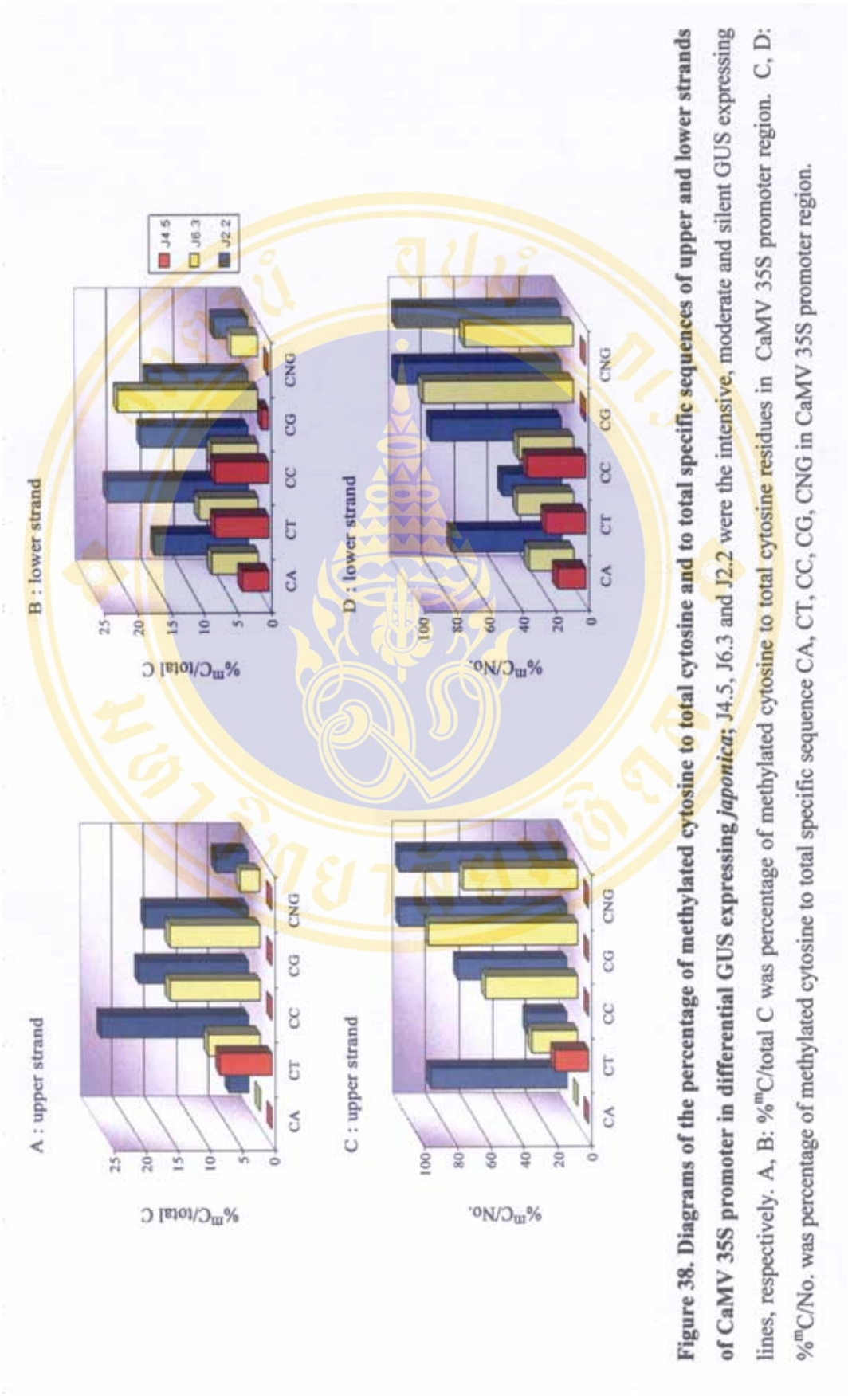

Figure 38. Diagrams of the percentage of methylated cytosine to total cytosine and to total specific sequences of upper and lower strands of CaMV 35S promoter in differential GUS expressing *japonica*; J4.5, J6.3 and J2.2 were the intensive, moderate and silent GUS expressing lines, respectively. A, B: %^{m}C/total C was percentage of methylated cytosine to total cytosine residues in CaMV 35S promoter region. C, D: %^{m}C/No. was percentage of methylated cytosine to total specific sequence CA, CT, CC, CG, CNG in CaMV 35S promoter region.

PART III Shoot apical meristem transformation in *indica* rice

1. Establishment of plant tissue culture system

A highly efficient and reproducible *in vitro* regeneration system is the most important step for producing transgenic plants, especially in monocot species. In this experiment, we used shoot apical meristem (SAM) as the plant material for transformation. The establishment of multiple shoot formation from shoot apical meristem was carried out by the effect of a synthetic urea-cytokinin, thidiazuron (TDZ). Rice seeds were dehusked, surface steriled and placed on MS medium containing 0, 1, 2, 4, 6, 8 mg/l thidiazuron (TDZ). Rice seeds were precultured for 2-3 days until the 0.5 to 0.7 cm seedlings were observed. At this stage, seedlings were separated from endosperms. Shoot tip and root were cut out, leaving about 1-mm long shoot before transferred to fresh medium. The shoot tips were cut out so as to increase the efficiency of multiple shoot formation. The seedlings gave rise to multiple shoots that could be observed within 2 weeks (Fig. 39A, B). There was no significant differences ($p = 0.05$) among 4, 6 and 8 mg/l TDZ for multiple shoot induction (Table 14). Thus, 4 mg/l TDZ was selected for use in the multiple shoot regeneration system. Three to four weeks later, multiple shoots were separated from each other and transferred to MS medium for root induction (Fig. 39C). The rice plants growing on MS medium showed no phenotypic variation (Fig. 39D).

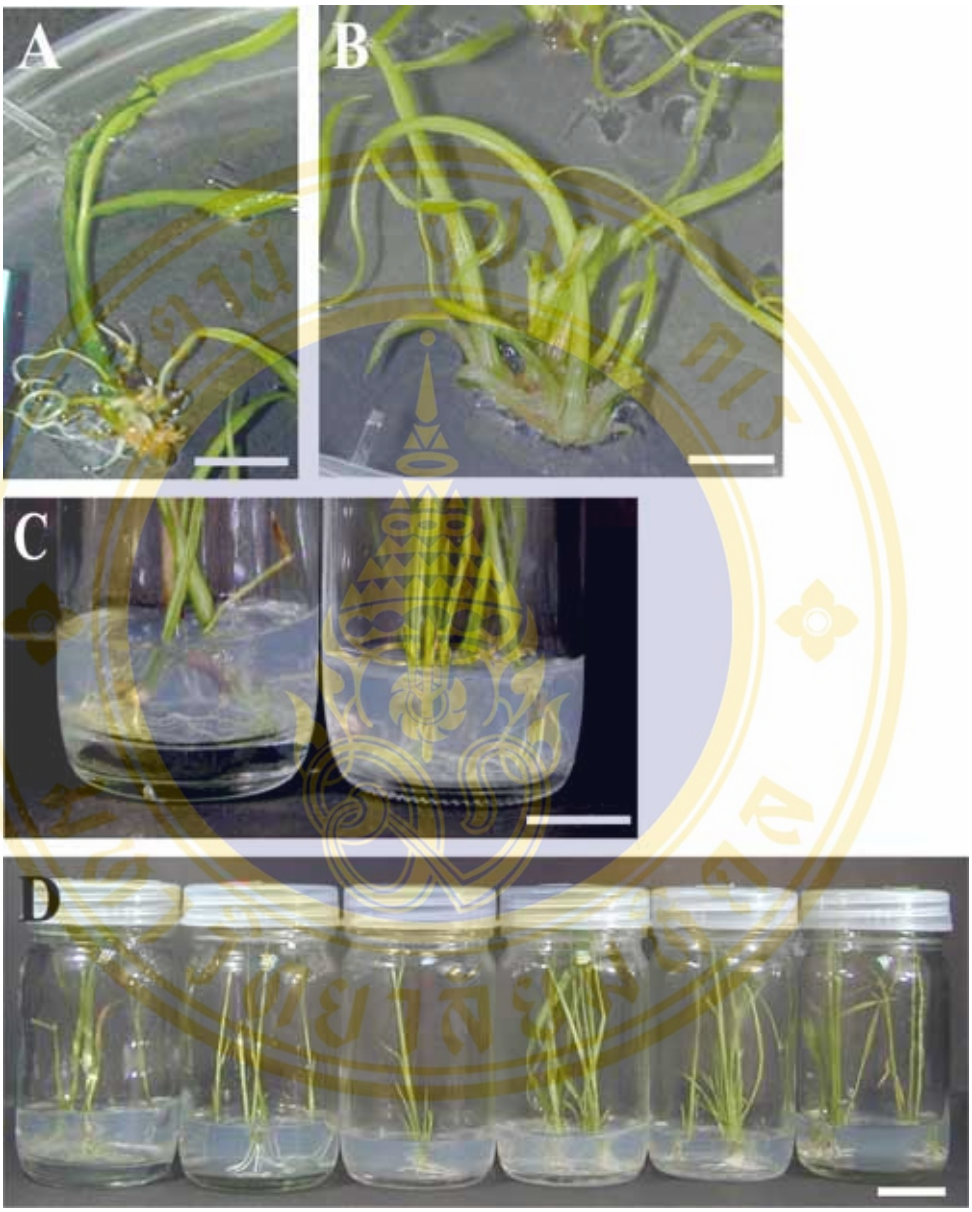

Figure 39. Induction of multiple shoot in *indica* rice (KDML105) by thidiazuron (TDZ)

(A) Single shoot formation of rice on MS medium as a control, (B) Multiple shoot formation of rice seedling grown on MS supplemented with 4 mg/l Thidiazuron (TDZ) for 2 weeks, (C) Root formation of rice plants obtained from (B) on MS medium, (D) .Three-weeks plants on MS medium. Bar = 2 cm.

Table 14. The number of rice shoots regenerated from shoot apical meristem on culture medium containing various concentrations of thidiazuron (TDZ)

TDZ (mg/l)	Number of shoots/meristem
0.0	1^{d}
1.0	5^{c}
2.0	6^{bc}
4.0	7^{ab}
6.0	8^{a}
8.0	7^{ab}

Means followed by a common letter was not significantly different (p = 0.05) by Duncan's multiple rang test (DMRT).

2. *Agrobacterium*-mediated transformation

2.1 Effect of hygromycin B, cefotaxime and carbenicillin on multiple shoot formation

To investigate the suitable concentrations of antibiotics used in *Agrobacterium*-mediated transformation of rice. Kill curves of hygromycin B, cefotaxime and carbenicillin were tested in rice seedlings. Shoot apical meristems were isolated from endosperms and placed on MS + 4 mg/l TDZ supplemented with 250 mg/l cefotaxime + 0, 50, 75, 100, 150 mg/l hygromycin B and 250 mg/l carbenicilin + 0, 50, 75, 100, 150 mg/l hygromycin B as described in materials and methods. The percentage of surviving shoots was observed every week for 1 month as shown in Fig. 40. From the experiment 2.2, 250 mg/l of cefotaxime and carbenicillin was the lowest concentration that inactivated bacterial growth. Therefore, in this experiment, we used the same concentration of cefotaxime and carbenicillin while different concentrations of hygromycin B were investigated. Using high concentrations of hygromycin B (> 50 mg/l), browning of plant tissues was observed within 2 weeks and all of them finally

died at the 3rd week. Thus, 50 mg/l hygromycin B was determined to be a minimum concentration with the ability to kill the rice seedlings. When the combination between cefotaxime/carbenicillin and hygromycin B was performed, the number of survival rice seedlings decreased rapidly within 2 weeks when cultured on carbenicillin containing medium. Therefore, we concluded that it would be better to use carbenicillin rather than cefotaxime under the same concentration (250 mg/l).

2.2 Study the viability of *Agrobacterium* on antibiotic supplemented medium

For transformation system, compatibility between *Agrobacterium* and plasmid vector is very important. We have to confirm that bacterial strain used and plasmid vector in an experiment are not resistant to the same antibiotics. All of antibiotics related to the transformation experiment were tested in *Agroacterium* to confirm the suitable concentrations for each strain. Thus, all of *Agrobacterium* strains; AGL1, AGL1 harboring pWBVec10a, AGL1 harboring pCAMBIA1301, EHA105 harboring pCAMBIA1301 and EHA101 were cultured on various antibiotic-supplemented medium (YEP medium containing 25, 50 mg/l rifampicin, 250 mg/l cefotaxime, 250 mg/l carbenicillin, 50 mg/l hygromycin B, 50 mg/l spectinomycin and 50 mg/l kanamycin) to observe their survival. The results were shown in Table 15. Consequently, when compared between 25 mg/l and 50 mg/l rifampicin, all of them could survive under the first concentration. Rifampicin resistance is the characteristic of *Agrobacterium* that separates it from other species. Thus we have to add this antibiotic in all of *Agrobacterium* cultured medium. Cefotaxime and carbenicillin, which interfer the synthesis of bacterial cell wall, are antibiotics used for inhibition of bacterial growth after gene transformation. While 250 mg/l cefotaxime could inhibit the growth of *Agrobacterium* strain AGL1, the strain EHA101 and EHA105 were could not survive in the medium with 250 mg/l carbenicilin. Kanamycin and Spectinomycin resistance are the characteristics of the plasmid pCAMBIA1301 (Fig. 8) and pWBVec10a (Fig. 9), respectively. To study the compatibility between *Agrobacterium* and the plasmid, the bacteria were grown on YEP medium supplemented with 50 mg/l spectinomycin and 50 mg/l kanamycin, respectively. In consequence, AGL1 strain did not survived on both antibiotics so this strain could be

used as a host of pWBVec10a and pCAMBIA1301. By contrast, resistance to these antibiotics was observed in strain EHA101. Therefore, we should not transform the plasmid vectors into this strain. Hygromycin B, a selective agent, is an aminocyclitol antibiotic that inhibits protein synthesis in both prokaryotes and eukaryotes. This ability is useful for plant transformation because the hygromycin resistant (*hpt)* gene is particularly effective in stringent selection of transformed plant cells. Resistance to hygromycin B was observed only in *Agrobacterium* that contained pCAMBIA1301 (AGL1-pCAMBIA1301 and EHA105-pCAMBIA1301) but not in wild type strains. In *Agrobacterium* that contained pWBVec10a, the viability could not be detected because of an intron insertion within the *hpt* gene coding sequence as shown in Fig. 9. The presence of CAT-1 intron in *hpt* gene in pWBVec10a is very useful in plant transformation. The addition of hygromycin B in selective medium is not only for selection of transgenic plants but it also can inhibit an overgrowth of *Agrobacterium* on plant tissues after transformation.

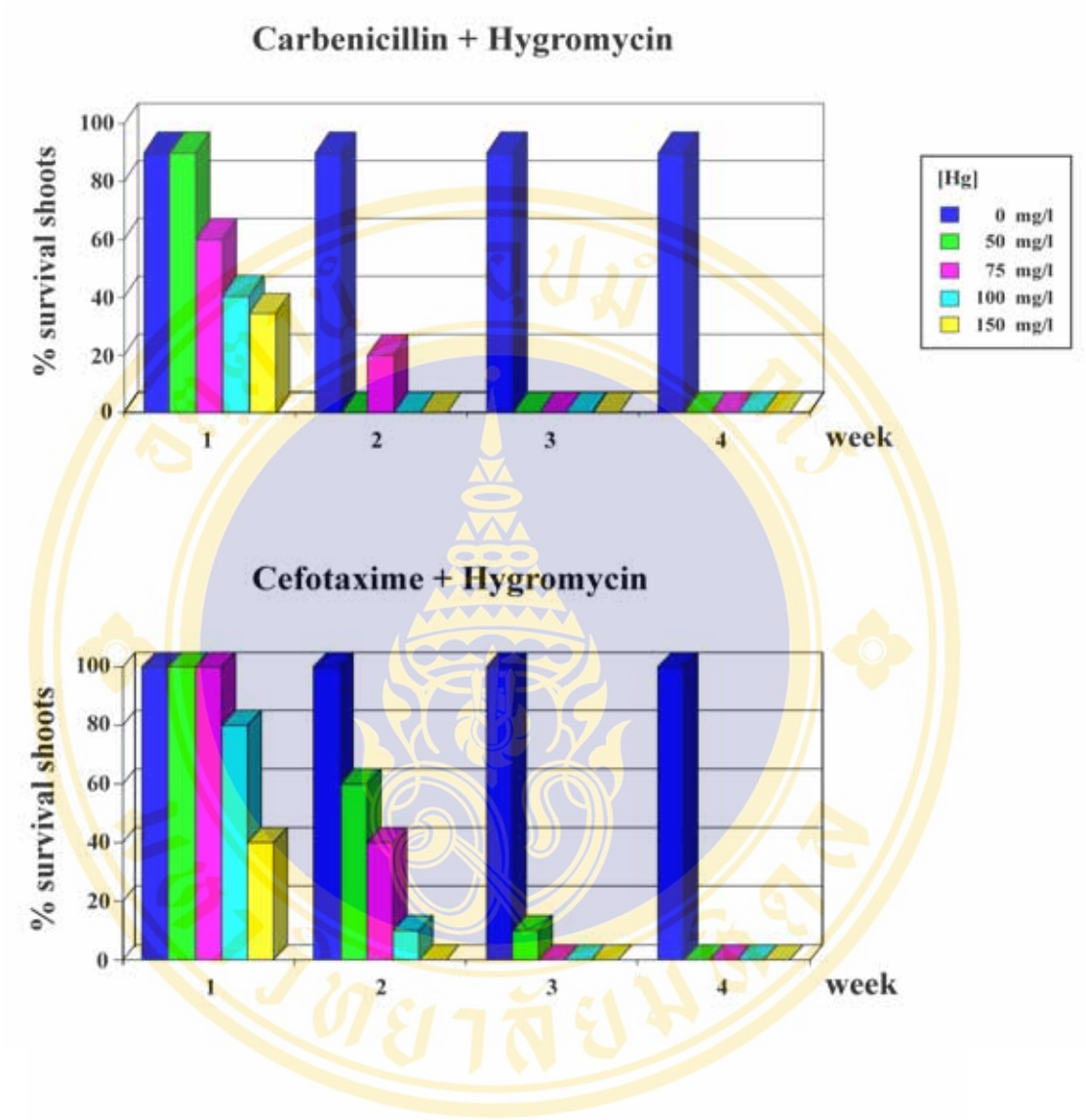

Figure 40. Effect of hygromycin B, cefotaxime and carbenicillin on multiple shoot formation

Shoot apical meristems of *indica* rice were cultured in (A) MS + 4 mg/l TDZ supplemented with 250 mg/l carbenicillin + 0-150 mg/L hygromycin B and (B) MS + 4 mg/l TDZ supplemented with 250 mg/l cefotaxime + 0-150 mg/l hygromycin B for 1-4 weeks. [Hg]: concentration of hygromycin B.

Table 15. Viability of *Agrobacteriums* on various antibiotic-supplemented medium

Antibiotic / Strain-plasmid	Rf		Cf	Cb	Spec	Km	Hg
	25 mg/l	50 mg/l	250 mg/l	250 mg/l	50 mg/l	50 mg/l	50 mg/l
AGL 1	+++	-	-	+	-	-	-
AGL 1-pWBVec10a	+++	-	-	+	+++	-	-
AGL 1-pCAMBIA1301	+++	-	-	+	-	+++	+++
EHA101	+++	-	+	-	+++	+++	-
EHA101- pWBVec10a	+++	-	+	-	+++	+++	-
EHA101-pCAMBIA1301	+++	-	+	-	+++	+++	-
EHA105-pCAMBIA1301	+++	-	+	-	-	+++	+++

Rf : rifampicin, Cf : Cefotaxime, Cb Carbenicillin, Spec : spectinomycin, Km : Kanamycin, Hg : Hygromycin B

2.3 Study the effect of acetosyringone in gene transformation

At first, *Agrobacterium* strain AGL1-pWBvec10a and EHA105-pCAMBIA1301 were used for gene transfer. However, low efficiency was detected in bacterial strain AGL1-pWBvec10a (data not shown). Therefore, we focused only on EHA105-pCAMBIA1301 for the transformation of shoot apical meristem of *indica* (cv. KDML105) rice. Transient expressions of GUS in the presence and the absence of acetosyringone (200 μM) were evaluated after bacterial inoculation and co-cultivation period. The frequency of GUS-expressing seedings under acetosyringone-challenged condition was 23% while without acetosyringone showed 22% positive seedlings (Table 16). We concluded that this chemical compound had no significantly effect on the transformation efficiency in *indica* rice.

2.4 Study the efficiency of gene transformation in various sonication times

To increase the efficiency of gene transformation, we studied the effect of sonication during bacterial infection. After the separation of shoot apical meristems from endosperms, they were immersed in bacterial suspension (EHA105-pCAMBIA1301) and sonicated at 0, 5, 10, 20 s. The level of GUS expression by histochemical analysis after 3-day co-cultivation was classified into two categories depending on the area of blue color in the transformed seedlings, <50% and >50% GUS-expressing area in seedlings. The results were shown in Table 17. Sonication at 20 s showed the highest (43±23%) number of the total GUS-expressing seedlings (combination of seedlings that contained <50% and >50% GUS-expressing areas) which was significantly different ($p = 0.05$) compared with 0 s. However, rice tissues were damaged under this condition and almost all of them could not survive when cultured on the selective medium. To reduce the chimera of transgene in transgenic plant that usually occurs in meristem transformation, the frequency of GUS-expressing seedlings that contained more than 50% of the blue area was examined. The highest frequency was observed at 10 s (8±5%) which was significantly different from 0 s but was not significantly different from 20 s. However, the seedlings that have been sonicated for 20 s were damaged. Therefore, sonication for 10 s during

Agrobacterium infection was the most suitable concentration to increase the transformation efficiency in shoot apical meristem of *indica* rice.

Table 16. The effect of acetosyringone in transformation efficiency of KDML105 using *Agrobacterium* EHA105-pCAMBIA1301

Experiment No.	% GUS-expressing seedling	
	+ Acetosyringone	- Acetosyringone
1	36	24
2	16	44
3	4	20
4	20	4
5	30	20
Average	**23**	**22**

Table 17. Percentage of GUS-expressing seedlings in KDML105 at different sonication times

Sonication time	%GUS-expressing seedling		
	+	++	Total
0 s	16±13[a]	1±2[a]	17±14[a]
5 s	28±3[a]	6±8[a]	14±8[ab]
10 s	28±10[a]	8±5[b]	37±14[ab]
20 s	36±21[a]	6±4[ab]	42±23[b]

Means followed by a common letter was not significantly different (p = 0.05) by Duncan multiple range test (DMRT).

\+ = < 50% of GUS-expressing area in seedlings

++ = > 50% of GUS-expressing area in seedlings

2.5 Establishment of stable transformation in shoot apical meristem

According to the study in 2.1 and 2.2, 50 mg/l hygromycin B and 250 mg/l carbenicillin were applied for selection of transgenic plants. Shoots were immersed in bacterial suspension, sonicated 10 s and co-cultivation for 3 days. After washing with 250-300 mg/l carbenicillin 3 times, a shoot tip was cut out, leaving around 2-3-mm long in order to increase the efficiency of multiple shoot formation. Explants were then cultured on MS-TDZ supplemented with 50 mg/l hygromycin B and 250 mg/l carbenicillin but the seedlings became brown and died within 2 weeks. Thus, we reduced the hygromycin B concentration to 25 mg/l for the selection. As a result, resistant shoots could be observed within 2 weeks (Fig. 41B, C and D). The 3-weeks regenerated rice was then transferred to MS containing 25 mg/l hygromycin B and 250 mg/l carbenicillin. We obtained two resistant plants from this system that were further analyzed by histochemical analysis. Blue color did not occur in both KA1 and KA2 resistant lines after incubation of leaf explants in X-Gluc solution. Molecular characterization and methylation status were investigated in the next experiments.

2.6 Molecular characterization and transgene integration analysis

2.6.1 PCR analysis

PCR amplifications were carried out in two resistant lines (KA1 and KA2) to confirm the presence of transgenes, the integration patterns and transgene rearrangements. *gus* gene, *hpt* gene, CaMV 35S-*gus* and CaMV 35S-*hpt* were observed only in KA1 (Fig. 42 and 43) implied that KA2 was not the transgenic. The presence of PCR products of the 4 regions confirmed the complete cassettes of *gus* and *hpt* genes, meaning that silencing of GUS in KA1 resulted from other factors not from transgene integration event. After observed the rearrangements (direct repeat and inverted repeat) by amplification with p1+1, p1+2 and p2+2 in KA1, no PCR products could be amplified from those 3 pairs of primers. This circumstance clarified that there was no tandem repeats between T-DNA insertions in KA1.

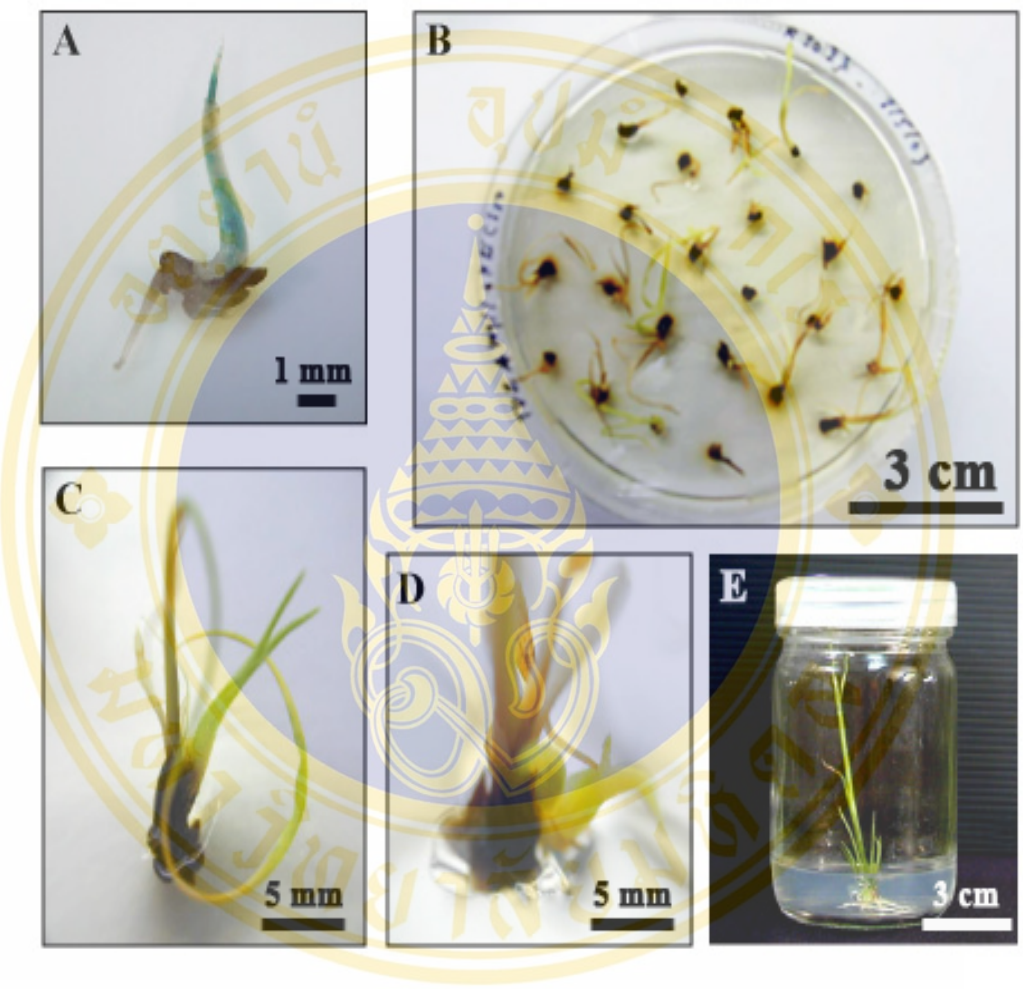

Figure 41. ***Agrobacterium*·mediated transformation in** ***indica*** **rice**

(A) transient expression of GUS in shoot apical meristem, (B, C, D) resistant shoots regenerated on MS supplemented with 4 mg/l TDZ and 25 mg/l Hygromycin B, (D) transgenic rice growing on MS medium supplemented with 25 mg/l Hygromycin B

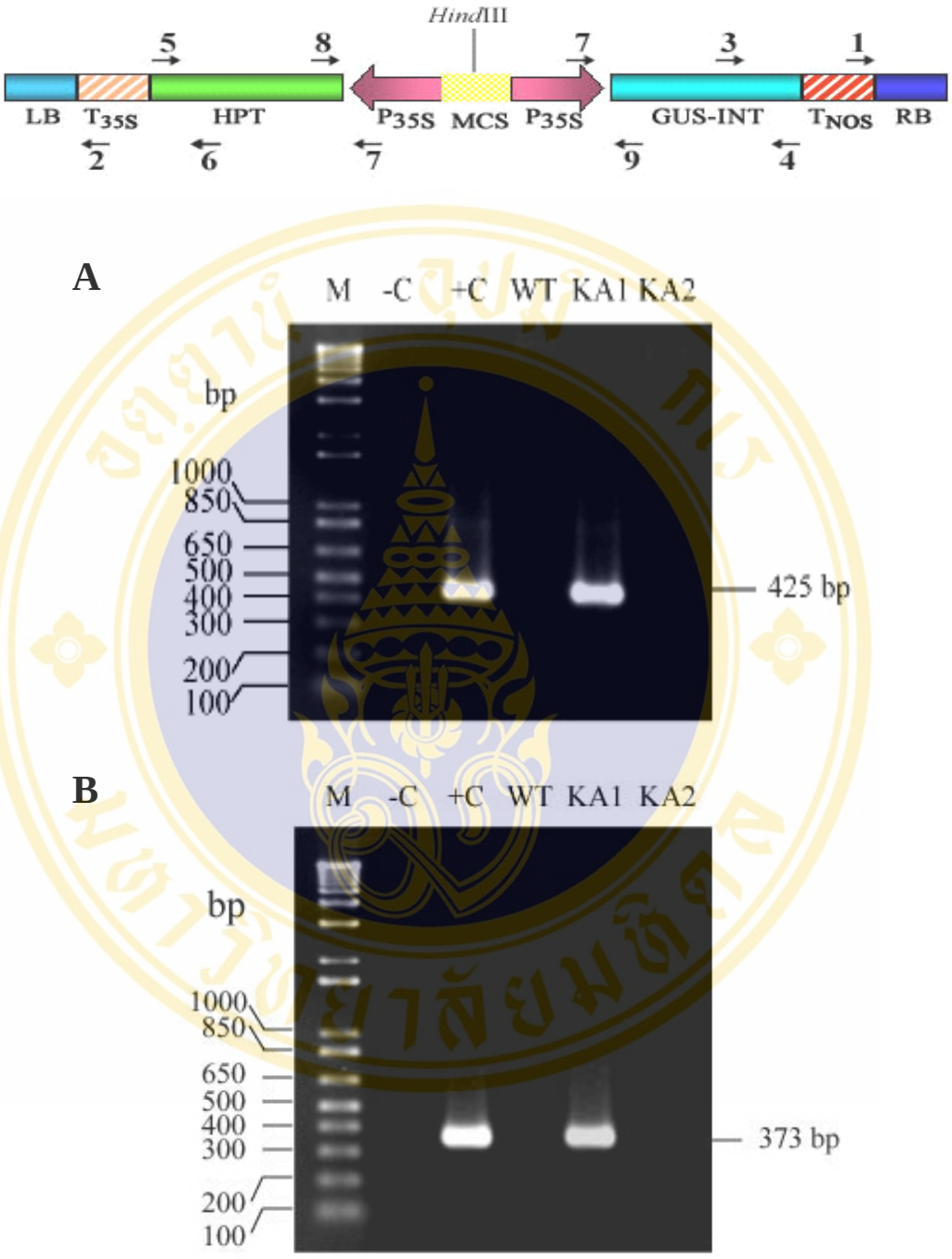

Figure 42. PCR amplification of the *gus* gene (p3+4) (A) and *hpt* gene (p5+6) (B) in *indica* rice using *Agrobacterium*-mediated transformation; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: plasmid pCAMBIA1301 as a positive control, WT: wild type plant, KA1 and KA2 were the regenerated plants grown on MS supplemented with 25 mg/l hygromycin B and 250 mg/l carbenicillin. Gel electrophoresis was performed on 1.5% (w/v) agarose.

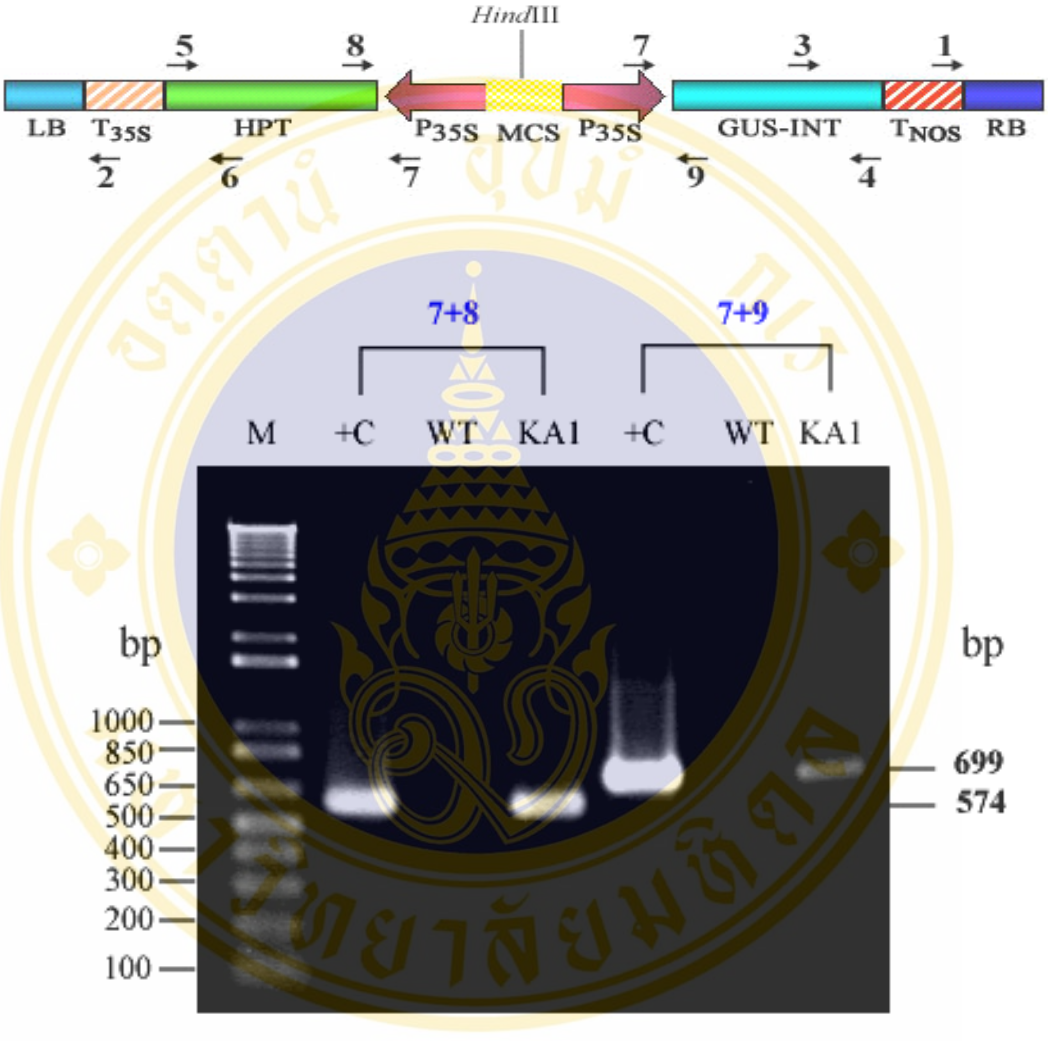

Figure 43. PCR amplification of CaMV 35S-*gus* (p7+9) and CaMV 35S-*hpt* (p7+8) regions in *indica* rice using *Agrobacterium*-mediated transformation; M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: plasmid pCAMBIA1301 as a positive control, WT: wild type plant, KA1 was the regenerated plants grown on MS supplemented with 25 mg/l hygromycin B and 250 mg/l carbenicillin. Gel electrophoresis was performed on 1.5% (w/v) agarose.

2.6.2 Southern hybridization

Southern hybridization was performed in KA1 to find out the copy number of *gus* gene as shown in Fig. 44. We observed 4 copies of *gus* in this transgenic line when hybridized with 425-bp radiolabeled probe that differ from other transgenic plants (tobacco and *japonica* rice) obtained from *Agrobacterium*-mediated transformation, which showed 1-2 copies. Hence, the increase of transgene copy number did not enhance the expression. On the other hand, this evidence may lead to an inactivation of the transgene.

2.7 Determination of *gus* expression by RT-PCR

Because we could not detect the GUS expression in KA1 from histochemical analysis, thus the investigation of *gus* transcript was also studied by RT-PCR. There was no expression of *gus* gene observed from this method (Fig. 45), so we concluded that silencing of GUS in transgenic rice (KA1) occurred from the absence of mRNA.

2.8 DNA methylation status analysis

Methylation of DNA at CaMV 35S promoter region was determined in KA1 plant using bisulfite genomic sequencing PCR technique as reported in transgenic tobacco and *japonica* rice. We discovered that the level of ^{m}C was similar to the silent transgenic *japonica* (J2.2). The percentage of methylated cytosine to total specific sequence CA, CT, CC, CG, CNG (%^{m}C/No.), showed that all of symmetric sequences, CG and CNG, in specific promoter region were methylated (Table 18 and Fig. 46). Moreover, the observation of asymmetric sequences revealed that methylation was frequently occurred at CA and CC but not CT.

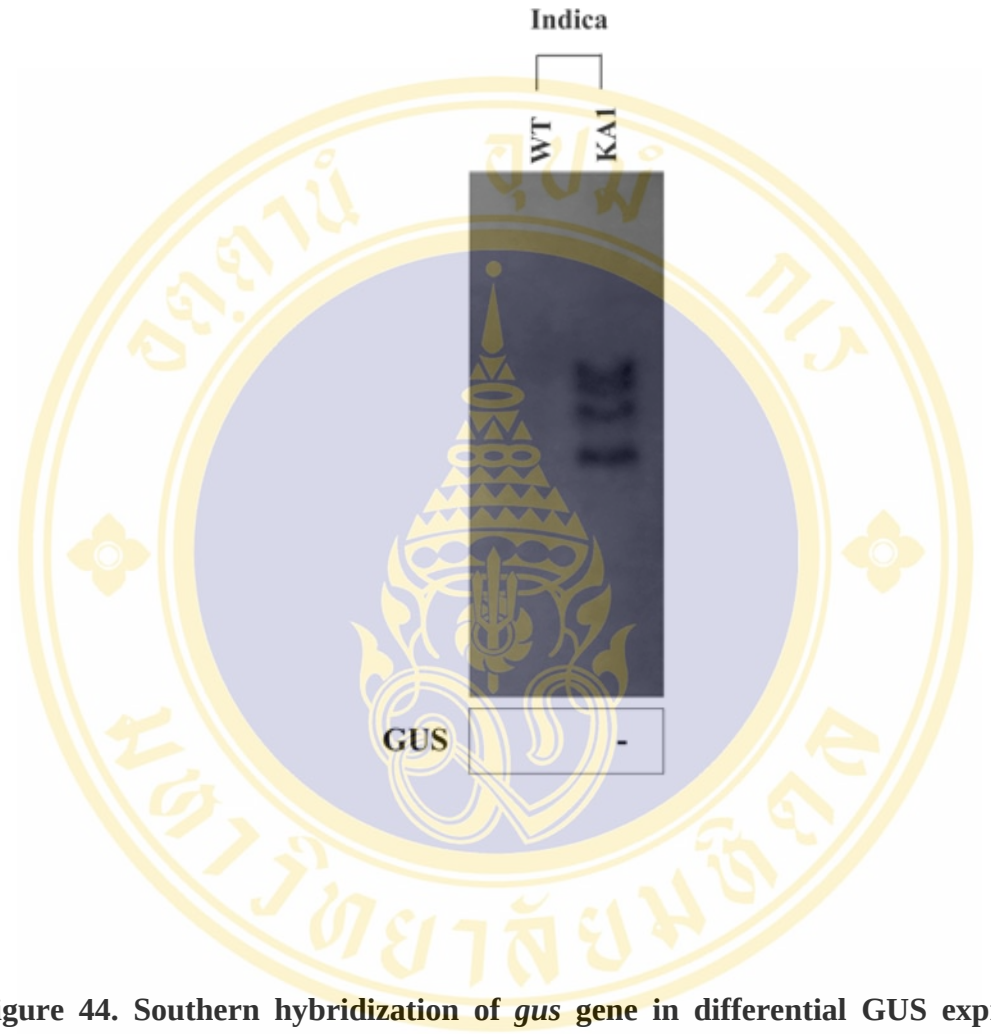

Figure 44. Southern hybridization of *gus* gene in differential GUS expressing lines of transgenic *indica* obtained from *Agrobacterium*-mediated transformation; the 425 bp fragment of *gus* was labeled with ^{32}P to be used as a probe.

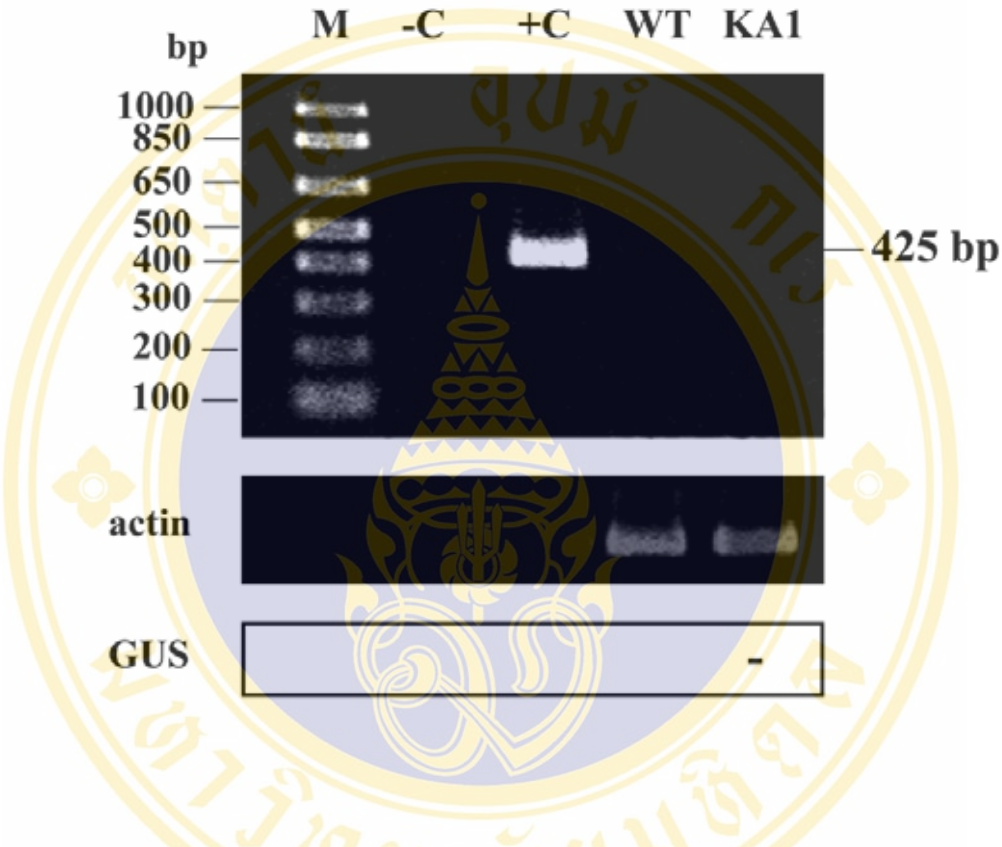

Figure 45. RT-PCR analysis of RNA transcribed from differential GUS expressing *indica* in 1.5% (w/v) agarose gel electrophoresis; the expression of *gus* transgene was not detected in transgenic line KA1. M: 100 bp DNA ladder (Invitrogen), -C: negative control, +C: pCAMBIA1301 as a positive control, WT: wild type plant. The *actin* gene expression was determined as a control of housekeeping gene.

Table 18. Percentage of methylated cytosine to total cytosine and to total specific sequences of upper and lower strand of CaMV 35S promoter region in transgenic *indica* rice line KA1

A: %^{m}C/total C was percentage of methylated cytosine to total cytosine residues in specific CaMV 35S region. Asymmetrical sequence were CA, CT, CC and symmetrical sequence were CG, CNG (N=A, T, C, G).

B: % ^{m}C/No. was percentage of methylated cytosine to total specific sequence CA, CT, CC, CG and CNG in CaMV 35S region.

(A)

%^{m}C/Total C	**Upper strand**	**Lower strand**
CA	3	14
CT	22	20
CC	16	18
Total asym. Seq	**41**	**52**
CG	15	24
CNG	5	10
Total sym. Seq	**20**	**34**

(B)

%^{m}C/No.	**Upper strand**	**Lower strand**
CA	81	65
CT	23	36
CC	63	78
CG	100	100
CNG	100	100

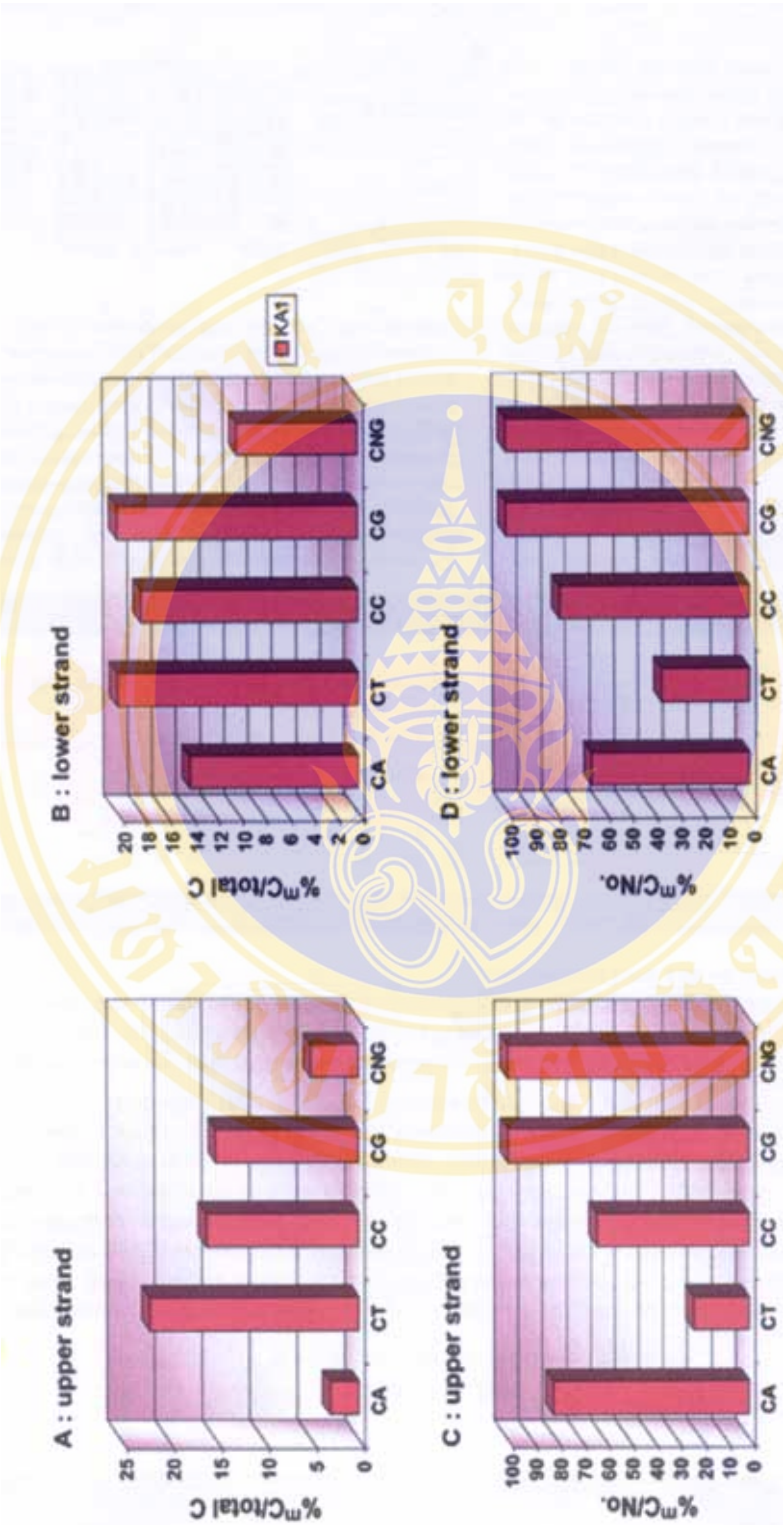

Figure 46. Diagrams of the percentage of methylated cytosine to total cytosine and to total specific sequences of upper and lower strands of CaMV 35S promoter in *indica* rice; line KA1 was the silent GUS expressing plant that contained the *gus* gene integration. A, B: %^{m}C/total C was percentage of methylated cytosine to total cytosine residues in CaMV 35S promoter region. C, D: %^{m}C/No. was percentage of methylated cytosine to total specific sequence CA, CT, CC, CG, CNG in CaMV 35S promoter region.

3. Particle bombardment

We have also tried to establish the system of particle bombardment in shoot apical meristem of *indica* (KDML105). After germination of mature seeds on TDZ-contained medium for 2-3 days, shoot apical meristems were separated from germinated seeds and placed on the same medium in a middle of the plate (50 meristems/plate) and precultured for 4 h before bombardment. A bombardment was carried out using the biolistic PDS-1000/He (Bio-Rad) Particle Delivery system. Various parameters involved in the efficiency of gene transformation were tested during bombardment. All of these parameters were acceleration pressure (1100, 1350 psi), target distance (9, 12 cm) and number of bombardments (1, 2 bombardment). The plasmid pCAMBIA1301 that contains *gus* and *hpt* genes under the control of CaMV35S promoter was coated with 1μm gold particle before bombarded into rice tissues at different treatments described above. After performed the experiments more than 5 times, we detected very few GUS-expressing seedlings (less than 1%) in the condition of 1,100 psi, 9-cm target distance and 1 time of bombardment (data not shown). GUS expression could not be observed in other conditions. Thus, optimization of bombardment in shoot apical meristem of *indica* rice should be investigated further. However, the interesting evidence found through the experiment was the effect of bombardment conditions on the regeneration of shoot apical meristems. The acceleration pressures, target distances and number of bombardments did not interfere with rice growth. Multiple shoot formations in the bombarded tissues and unbombarded tissue (control) on the non-selective medium were not different.

CHAPTER 5
DISCUSSION

1. Shoot apical meristem as an alternative material for gene transfer in *indica* rice

Establishment of high efficiency *Agrobacterium*-mediated transformation has greatly facilitated the widespread application of transformation in *japonica* rice (*Oryza sativa* ssp. *japonica*) (5). Currently, this technique is widely used not only to introduce genes of interest into the rice genome for the purpose of varietal improvement (198-200), but also as a common means for testing gene function by enhancing or inhibiting expression of target genes (201-203). On the contrary, there have been very few successful reported of transformation in *indica* rice (*Oryza sativa* ssp. *indica*). Even in reports in which transformation succeeded, the results showed either low transformation efficiency (204-206), or success only with very specific genotypes (207). Several publications reported about transformation of *indica* rice using embryonic callus as a plant material (204, 206, 207); however, robust and widely applicable methods for subculture and regeneration have been the main limitations. Transgenic plants regenerated through a more-or-less long-term callus phase have an increased risk of somaclonal variation, problem in transgene inheritance and stability of transgene expression (208). Therefore, in our study, we have established the system of shoot apical meristem (SAM) as an alternative material for gene transfer in *indica* rice (*Oryza sativa* ssp. *indica* cv. KDML105). It was observed that regenerated plants obtained from shoots provided the low incidence of somaclonal variation (105). Shoot apex has been employed in either *Agrobacterium*-mediated transformation or particle bombardment in many plant species (108-111, 209, 210). However, recover of transgenic plants directly from shoot apical meristem will always be chimera (113). Thus, the possibility for avoidance this phenomenon is the multiplication of transgenic meristem cells by plant growth regulator treatments (113).

Thidiazuron (*N*-phenyl-*N'*-1,2,3-thidiazol-5-ylurea; TDZ) is one of several substituted ureas that has been investigated for cytokinin activity, TDZ has been reported to facilitate multiple shoot proliferation of many plant species (115, 211-214). At 10 μM, TDZ was better than BA for inducing shoot regeneration of rice (*Oryza sativa* spp. *indica* cv. Jaumala) embryonic callus (114). It is active at lower concentrations than the amino purine cytokinins and found to be less susceptible to plant degradating enzymes than endogenous cytokinins (215). Morover, plant regeneration can be stimulated though exposure to TDZ for a relatively short time (216). The different potential modes of action of TDZ have been reviewed by Murthy *et al.* (213). TDZ may be involved in the reprogramming and expression of the competent cells necessary for them to undergo differentiation and development. TDZ was found to induced synthesis or accumulation of endogenous cytokinins because of an increase in synthesis, a decrease in catabolism, or a conversion of storage forms to biologically active cytokinins (217-219). In our experiment, various concentrations of TDZ were tested for multiple shoots formation from rice shoot apical meristems. As a result, we found that 4 mg/l of TDZ was the most suitable concentration that gave rise to 7 shoots/meristem. Increasing of TDZ concentration the 6 and 8 mg/l did not increase the formation of rice shoots. Furthermore, rice plants regenerated from this system showed normal development as well as the germination on medium lacking of the growth regulator.

Agrobacterium strain AGL1 harboring the plasmid pWBvec10a and EHA105 haboring the plasmid pCAMBIA1301 were employed for gene transfer. Although both AGL1 and EHA105 strains harbor a hypervirulent Ti plasmid and exhibit a broad range of high transformation frequency (220), the latter was more effective for transformation of KDML105, the result was similar in transformation of tobacco and *japonica* rice in our research. Recently, EHA105 has been reported as the most suitable *Agrobacterium* strain in *japonica* and *indica* rice transformation compared with the strain LBA4404 and AGL1 (221). The similar evident in transformation of *Digitalis minor* (222) showed the high transformation efficiency (8.4%) when using the EHA105 strain while experiments conducted with the strain AGL1 resulted in partial failure. Therefore, EHA105 was respected to gene transformation in other experiments of our study.

In order to enhance transformation rate, the effect of acetosyringone and sonication were investigated. The addition of acetosyringone during co-cultivation has been reported for the successful transformation in callus of *indica* rice (207) and *javanica* rice (7). In contrast, our results revealed that addition of 200 μM acetosyringone did not significantly increase the transformation efficiency in shoot apical meristem of KDML105. Recently, it has been reported that sonication can be used to enhance *Agrobacterium*-mediated transformation of many different plant species (223-226). The enhanced transformation rates using sonication while immersed in an *Agrobacterium* suspension probably result from microwounding, where the energy released by cavitation causes small wounds both on the surface of and deep within the target tissue (227). Unlike callus, shoot apical meristem contains several layers of specialized cells packed together which cannot be easily infected by *Agrobacterium* thus the increase of wounding by sonication should be a good physical treatment to enhance the transformation efficiency. As a result, the increase of sonication period led to the higher transient expression. However, a treatment up to 20 s resulted in severe tissue disruption and death, so that we preferred the 10-s sonication period for shoot apical meristem transformation of KDML105.

Because of the tissue damages from sonication and *Agrobacterium* infection, the transformed apices turn brown and fail to elongate further when cultured on the selective medium containing 50 mg/l hygromycin B in the first set of transformation experiment. Thus we reduced the antibiotic concentration to 25 mg/l to select the transgenic plants. Transformed apical meristem generated green resistant multiple shoots around 1-3 shoots/meristem within 2 weeks; however, after the multiple shoots were separated from the seedling and transferred to MS supplemented with 25 mg/l hygromycin B for root induction, only few plants were obtained. Although hygromycin phosphotransferase (*hpt*), which confers resistance to the amino glucoside antibiotic hygromycin, has been reviewed by Hiei *et al*. (228) as an efficient marker for selection of transgenic rice after *Agrobacterium*-mediated transformation, it should be note that the optimization of antibiotic concentration during stable selection is very important and diverse in different plant species. For instance, the recovery of transgenic cassava (*Manihot esculenta* Crantz) was carried out by gradually increase hygromycin concentrations (0, 7.5, 15 mg/l) and decrease again to be 10 mg/l for

shoot primordia development during selection of the transgenic (229). In our point of view, successful selection of transgenic KDML105 using multiple shoot induction system should be investigated further so as to optimize the most suitable concentration of hygromycin and the duration of selection.

The development of methods for gene transfer of monocotyledonous plants was delayed for some time as compared to the initial success in dicotyledonous species which are routinely transferred by the method of *Agrobacterium*-mediated transformation. Therefore, the direct gene transfer technique such as particle bombardment has been preferred as the most popular one especially in cereal plants (198). In rice, Christou *et al.* (230) published the report of the development of a variety-independent method based on an electric discharge particle acceleration of immature embryos that led to the recovery of fertile transgenic plants from many varieties, both *indica* and *japonica* at high frequencies. Successful transformation by particle bombardment for *indica* rice utilizing embryonic callus derived from mature-seed explants has been reported in 1998 (231). Various bombardment parameters were optimized to increase the efficiency of transformation events and transgenic rice plants were recovered as described elsewhere (231). However, it has no evidence of the transformation in shoot apical meristem of *indica* rice. In our study, we have tried to establish the particle bombardment transformation in shoot apical meristem of KDML105 using multiple shoot regeneration system. Various bombardment parameters including acceleration pressures, target distances and number of bombardments were examined. Particle acceleration has an important influence on the distribution of particles. Low acceleration pressures (650-1,100 psi) resulted in larger areas being covered by particles than higher acceleration pressures (1,300 and 1,550 psi) (232). Microscopic analysis of bombardment in wheat callus showed that at low pressures expression events were evenly distributed and at a relatively lower density, thus reducing bombardment shock and tissue injury. On the contrary, at high pressure, a small area of the target tissues was very strongly targeted and thus likely to be damaged (232). Multiple bombardments have also been discussed in wheat tissues with no significant difference in GUS expression but increased the damage of target tissues.

From our results, transformation efficiencies were rarely low in all experiment conditions. Transient expression of GUS was detected less than 1% in the condition of 1,100 psi, 9-cm target distance and 1 time of bombardment. It is not clear why the GUS activity was undetectable in other conditions. In our view point, the morphology of the explants utilized during the bombardment process is possibly a main factor for the successful transformation. Moreover, shoot apical meristem composes of many layers so we are not known how deep the particles should penetrate in order to reach the cells that could generate transgenic plants. The different parts of shoot apical meristem have been divided in layers (L1, L2 and L3) (see histology of shoot apical meristem in Fig.4). Layer L1 is the most external and forms the epidermis of the differentiated regions. Layers L2 and L3 divide preferentially in the anticlinal and periclinal planes to form the organs. The differentiated *de novo* shoots originate from the sub-epidermal layers (L2 and L3) of the apical meristem in the peripheral regions of the apical meristem (103). Although the high acceleration pressure and the multiple bombardments have been discussed for severe damage of plant tissue (232), we found that these parameters did not affect the multiple shoot regeneration in shoot apical meristem of KDML105. The bombarded explants grew as fast as the unbombarded ones under the multiple shoot regeneration condition without an antibiltic stress. Thus, the high efficiency of this tissue culture system should be a new alternative source for particle bombardment transformation in *indica* rice.

2. Gene transfer technique, transgene integration patterns and its expression

The stable expressions of foreign genes are of critical importance in the application of genetically engineered plants to agriculture. Hence, we have studied the correlation of transgene expression and transgene integration patterns in transgenic tobacco obtained from *Agrobacterium*-mediated transformation and particle bombardment. Moreover, using *Agrobacterium*-mediated transformation, the investigations have been carried out in transgenic *japonica* (*Oryza sativa* spp. *japonica* cv. Taichung) using embryonic callus as a source for gene transfer, and transgenic *indica* (*Oryza sativa* ssp. *indica* cv. KDML105) using shoot apical meristem as a plant material incorporated with the multiple shoot regeneration system.

In transgenic tobacco plants, detection of GUS activity from histochemical analysis revealed the evidence of gene silencing up to 70% through particle bombardment whereas 100% of transgenic tobacco plants obtained from *Agrobacterium*-mediated transformation showed GUS positive. The results from PCR analysis accounted for the missing of GUS activity of bombarded plants. We observed the deletions of CaMV 35S region and *gus* gene in all silent transgenic tobacco plants. The missing of *hpt* gene might also be occurred but we did not observe because the transgenics were selected under hygromycin-stressed condition so that plant tissues lacking of *hpt* cassette were fail to recover on antibiotic-contained medium. Similar evident was reported in orchid by Men *et al.* (233). In particle bombardment, transgenes are delivered to plant cells by physical means. DNA is precipitated onto the gold particles, a process in which a vortex is used, and then the DNA-coated particles are delivered to plant cells at high pressure and high velocity. During these processes, the DNA might be sheared, and this may be one of the reasons for separate integration and deletion of the transgenes (233). Furthermore, the high accelerated pressure during bombardment might be another factor of plasmid shearing that brings about the incomplete integration of transgene cassette in plant genome. When compared between *Agrobacterium*-mediated transformation and particle bombardment methods, the latter resulted in gene silencing rather than the former one. Although silencing of *gus* was also discovered in transgenic *japonica* rice obtained from *Agrobacterium*-mediated transformation, the percentage of silencing (57%) was lower than that of bombarded tobacco plants.

While the complete integration of T-DNA was observed in all transgenic lines of tobacco which were derived from *Agrobacterium*-mediated transformation, the loss of CaMV 35S promoter region and/or *gus* gene were presented in 13 of 30 transgenic *japonica* rice . Undetectable of intact copy of the *gus* gene in transgenic rice mediated by *A. tumefaciens* was reviewed by Hiei *et al.* (228). Thirteen out of twenty rice plants transformed with LBA4404 (pTOK233) that were negative in a histochemical analysis resulted from the deletion of *gus* transgene. Published results indicate that transgene silencing is more prevalent in monocotyledonous than in dicotyledonous plants but this may be an artifact of the predominant use of direct DNA uptake approaches to monocot transformation versus the widespread use of *Agrobacterium*-mediated

transformation of dicots (234). Nevertheless, our studies revealed that under the same method of transformation, the variation of T-DNA integration patterns in monocotyledonous plant (rice) seems to be higher than dicotyledonous plant (tobacco).

Transgene rearrangement, multiple copies of transgene or even additional copies of an endogenous gene have been reported as the reasons of gene silencing (21, 22). Differential expression of *gus* gene in the "complete-T-DNA-integration" transgenic plants obtained from *Agrobacterium*-mediated transformation (tobacco, *japonica* and *indica* rice) were classified into 3 levels; silent, moderate and intensive GUS expression. The results from Southern hybridization showed that copy numbers of transgene did not have any correlation with the *gus* expression levels since both one and two copies of *gus* led to the moderate and high GUS activities. On the contrary, silencing of *gus* gene could be detected in either one or four copies. It has been reported that, the variation of *gus* expression levels in transgenic barley did not correlate with copy number but the most likely reason for such correlated levels was a position effect of transgene in the chromosomal locations (235).

The rearrangements of transgene in direct repeat and inverted repeat formations have been evaluated using PCR amplification. However, all of the transgenic plants showed negative results. Southern hybridization in delegate transgenic plants revealed that almost all of them had single copy so that we could not detect any rearrangement events. Two and four transgene copies were observed in some plants but they might be located in different locus of plant chromosome thus it was not possible to achieve PCR amplification.

3. DNA Methylation at CaMV 35S promoter region revealed the transcriptional gene silencing (TGS)

According to the results from RT-PCR, the levels of *gus* transcripts in transgenic plants that contained the complete cassette of transgene decreased linearly from the intensive GUS-expressing plants to the silent plants. These phenomena raise our hypothesis that whether the silencing of *gus* transgene occurred at the transcriptional

level; hence, the investigation of cytosine methylation in promoter region was carried out in transgenic tobacco and rice plants.

Plant genomes contain substantial amounts of 5-methylcytosine. Up to 2-30% of the cytosines are methylated in the nuclear genome of many flowering plants (angiosperms). Much of this modification is found in the short symmetrical sites, CG and CNG, but non-symmetrical methylation outside of these sites is also found in angiosperm genomes (236). It is possible to maintain this modification at symmetrical CNGs through subsequent rounds of DNA replication throughout the activity of so-called maintenance DMTases. These enzymes can recognize methylated C in the template strand and catalyze methylation of the opposite C in the newly synthesized strand (25). The traditional maintenance activity for CG dinucleotides is Dnmt1; the plant homolog is MET1 (123, 124, 129). There is no known maintenance activity for asymmetrical CNN (where N is not G) nucleotide groups. Therefore, if methylation is observed in asymmetrical Cs, this can be taken as a measure of ongoing *de novo* methylation (156, 160).

In our experiments, using the bisulfite genomic sequencing PCR, the estimation of overall of methylation in CaMV 35S promoter of DNA from transgenic tobacco plants, *japonica* rice and *indica* rice showed the significant increase of DNA methylation in relation to transgene regulation at symmetrical and asymmetrical sequences when the *gus* transgene turn silent. These evidences revealed that promoter methylation either on symmetrical or asymmetrical sequences were precisely crucial for transgene inactivation at the transcription level. Meyer *et al*. (237) observed about 7% of symmetrical CG sites methylated in the active 35S promoter, whereas in an inactive one, this level reached 97%. Mosaic methylation was also observed in a mutant 35S promoter deficient for symmetrical methylation sites, and the levels were significantly increased in a silenced state (238). It has been reported that methylation of the first untranslated exon and 5′ region of the intron of the *Ubi1* promoter complex are correlated with transcriptional gene silencing in barley, and, incorporated with the condensation of chromatin in transgene containing regions (239). In rice, transformation of pUbi1-bar indicated that TGS was associated with methylation of the 3′ region of the core promoter (240). Heavy methylation of both symmetrical and nonsymmetrical sites of CaMV 35S promoter has been reported in tobacco (241).

After 24 months of callus *in vitro* cultivation, an epigenetic variant, designated locus 1E, was obtained in which cytosine methylation of symmetrical (CG and CNG) sites was almost complete within the 5′end of the *nptII*-transcribed region and the CaMV 35S promoter. Further, methylation of nonsymmetrical sites appeared *de novo* in the promoter (241). A pattern similar to that have also been found in a transcriptionally silenced CaMV 35S promoter in other transgenic lines (237, 242). Thus, from our research we concluded that the silencing of transgene resulted from the integration events during transformation and the cytosine methylation at promoter region which confirmed the transcriptional gene silencing in both dicotyledonous and monocotyledonous plants.

CHAPTER 6
CONCLUSION

The ultimate goal of this research was to study the transgene integration and promoter methylation in transgenic tobacco and rice that correlate with differential transgene expression. From all of the experiments through this research, we could summarize as following:

1. Gene transformation in tobacco

1.1 From *Agrobacterium*-mediated transformation, we used the bacterial strain EHA105 harboring pCAMBIA1301 to transfer gene into leaf disks of tobacco (*Nicotiana tabacum* cv. Samson NN). Eighteen percent of resistant calli were recovered and resistant shoots were regenerated with an average of 6 shoots/callus. All of transgenic plants maintained GUS activities which were classified into 2 categories; moderate GUS-expressing plants and intensive GUS-expressing plants.

1.2 From particle bombardment, we used pCAMBIA1301 as a vector model. Resistant calli were observed with an average of 7%. Tobacco shoots were regenerated with an average of 1.42±0.53 shoots/resistant callus. The results from histochemical analysis reveled that only 30% of putative transgenic plants maintained GUS activities.

2. Gene transformation in *japonica* rice

Calli of *japonica* rice (*Oryza sativa* ssp. *japonica* cv. Taichung) were transformed by *Agrobacterium* strain EHA105 harboring pCAMBIA1301. The frequency of putative transgenic plants/resistant callus was 5±2.19. The number of GUS-expressing plants was 43%. Differential patterns of GUS staining were characterized into three levels: silent, moderate and intensive GUS-expressing plants.

3. Shoot apical meristem transformation as an alternative for gene transfer in *indica* rice

3.1 Shoot apical meristem (SAM) of *indica* rice (*Oryza sativa* ssp. *indica* cv. KDML105) was employed as a plant material for gene transfer because regenerated plants obtained from shoot apices provide the low evident of somaclonal variation as occurred in callus tissue. However, recover of transgenic plants directly from shoot apical meristem will always be chimera. Therefore, the multiple shoot regeneration system was the alternative way to avoid this phenomenon. Using 4 mg/l of the synthetic urea-cytokinin, thidiazuron (TDZ), multiple shoots of KDML105 were induced. The fast-growing and healthy rice plants were obtained with an average of 7 shoots/explant, and no evidence of abnormal phenotype.

3.2 *Agrobacterium*-mediated transformation

3.2.1 Fifty mg/l hygromycin B was determined to be a minimum concentration for selection system. Under the same concentration (250 mg/l), carbenicillin had more effect on multiple shoot formation than cefotaxime.

3.2.2 Viability of *Agrobacteriums* on various antibiotic-supplemented medium was investigated as summarized in Table 15.

3.2.3 Although both *Agrobacterium* strain EHA105 and AGL 1 harbor a hypervirulent Ti plasmid and exhibit a broad range of high transformation frequency, the former was more effective for gene transfer of KDML105.

3.2.4 To enhance transformation rate, the effect of acetosyringone and sonication were investigated. As a result, acetosyringone had no effect on transformation efficiency whereas using sonication during the immersion of explants in bacterial suspension for 10 s significantly enhanced the transformation efficiency. It was due to the microwounding of rice tissue both on the surface of and deep within the target tissue.

3.2.5 As a consequence of bacterial infection and sonication, rice explants could not survive under the selective condition of 50 mg/l hygromycin B; hence, we reduced the concentration of hygromycin B to 25 mg/l for selection of transgenic plants. Even though the transformants generated green resistant multiple shoots within 2 weeks, almost all of them fail to survive on root induction medium under the stress condition.

3.2.6 Only one transgenic plant was obtained with no activity of GUS by histochemical analysis. Thus the selection system should be investigated further for an efficient transformation system in shoot apical meristem of *indica* rice.

3.3 Particle bombardment

Various parameters including acceleration pressures, target distances and number of bombardments were examined because they might affect the distribution of DNA coated particles during bombardment and cause the damage of plant tissues. Although very few GUS-expressing seedlings (less than 1%) were obtained only under the condition of 1,100 psi, 9 cm target distance and 1 time of bombardment, we found that all of the parameters did not affect the multiple shoot regeneration. Hence, the high efficiency of this tissue culture system should be a new alternative source for particle bombardment transformation in *indica* rice.

4. Gene transfer techniques, transgene integration patterns and its expression

4.1 In transgenic tobacco, while the plants obtained from *Agrobacterium*-mediated transformation gave 100% of GUS activity, silencing of *gus* transgene was detected up to 70% in tobacco plants derived from particle bombardment. The reason of gene silencing was revealed by PCR analysis, consequently, the missing of CaMV 35S region and *gus* gene were detected in all silent lines. Deletion of transgene in particle bombardment might be due to the shearing of DNA during precipitation of DNA onto gold particles and/or the high accelerated pressure during bombardment process.

4.2 Undetectable of an intact copy of *gus* gene was also observed in transgenic *japonica* rice. We concluded that under the same method of transformation (*Agrobacterium*-mediated transformation), the variation of T-DNA integration patterns in monocotyledonous plant (rice) was higher than dicotyledonous plant (tobacco).

4.3 Southern blot hybridization revealed that copy numbers of transgene did not have any correlation to *gus* expression.

4.4 Transgene rearrangements were not observed in all transgenic plants. It was possibly due to the single-copy integration and the spatial effect incase of multiple transgene integrations.

4.5 RT-PCR analysis showed that, the levels of *gus* transcripts decreased from the intensive, moderate and silent GUS-expressing plants, respectively. These outcomes supported the transcriptional gene silencing.

5. DNA methylation at CaMV 35S promoter region revealed the transcriptional gene silencing (TGS)

Using the bisulfite genomic sequencing PCR, the estimation of DNA methylation in CaMV 35S promoter from transgenic tobacco, *japonica* rice and *indica* rice showed the significant increase of methylated cytosines when the transgene turn silent. These investigations verified that the methylation either on symmetrical or asymmetrical sequences were precisely crucial for transgene inactivation. Therefore the silencing of transgene not only resulted from transgene integration patterns, but also from the inactivation of transgene at transcription level by promoter methylation.

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APPENDIX

APPENDIX A

The T-DNA sequence of pCAMBIA1301 from LB to RB

LB: 8623-8648, *hpt* gene: 8942-9964, CaMV 35S promoter of *hpt* gene: 9960-10807, CaMV 35S promoter of *gus* gene: 11294-11831, *gus* gene: 4-2095, RB: 2364-2389

Sequences in the boxes are the locations of primers (see more detail in table 5).

```
8601  CGGGGAGCTG TTGGCTGGCT GGTGGCAGGA TATATTGTGG TGTAAACAAA
      GCCCCTCGAC AACCGACCGA CCACCGTCCT ATATAACACC ACATTTGTTT
8651  TTGACGCTTA GACAACTTAA TAACACATTG CGGACGTTTT TAATGTACTG
      AACTGCGAAT CTGTTGAATT ATTGTGTAAC GCCTGCAAAA ATTACATGAC
8701  AATTAACGCC GAATTAATTC GGGGGATCTG GATTTTAGTA CTGGATTTTG
      TTAATTGCGG CTTAATTAAG CCCCCTAGAC CTAAAATCAT GACCTAAAAC
8751  GTTTTAGGAA TTAGAAATTT TATTGATAGA AGTATTTTAC AAATACAAAT
      CAAAATCCTT AATCTTTAAA ATAACTATCT TCATAAAATG TTTATGTTTA
8801  ACATACTAAG GGTTTCTTAT ATGCTCAACA CATGAGCGAA ACCCTATAGG
      TGTATGATTC CCAAAGAATA TACGAGTTGT GTACTCGCTT TGGGATATCC   PCAM-1
8851  AACCCTAATT CCCTTATCTG GGAACTACTC ACACATTATT ATGGAGAAAC
      TTGGGATTAA GGGAATAGAC CCTTGATGAG TGTGTAATAA TACCTCTTTG
8901  TCGAGCTTGT CGATCGACAG ATCCGGTCGG CATCTACTCT ATTTCTTTGC
      AGCTCGAACA GCTAGCTGTC TAGGCCAGCC GTAGATGAGA TAAAGAAACG   PCAM-5
8951  CCTCGGACGA GTGCTGGGGC GTCGGTTTCC ACTATCGGCG AGTACTTCTA
      GGAGCCTGCT CACGACCCCG CAGCCAAAGG TGATAGCCGC TCATGAAGAT
9001  CACAGCCATC GGTCCAGACG GCCGCGCTTC TGCGGGCGAT TTGTGTACGC
      GTGTCGGTAG CCAGGTCTGC CGGCGCGAAG ACGCCCGCTA AACACATGCG
9051  CCGACAGTCC CGGCTCCGGA TCGGACGATT GCGTCGCATC GACCCTGCGC
      GGCTGTCAGG GCCGAGGCCT AGCCTGCTAA CGCAGCGTAG CTGGGACGCG
9101  CCAAGCTGCA TCATCGAAAT TGCCGTCAAC CAAGCTCTGA TAGAGTTGGT
      GGTTCGACGT AGTAGCTTTA ACGGCAGTTG GTTCGAGACT ATCTCAACCA
9151  CAAGACCAAT GCGGAGCATA TACGCCCGGA GTCGTGGCGA TCCTGCAAGC
      GTTCTGGTTA CGCCTCGTAT ATGCGGGCCT CAGCACCGCT AGGACGTTCG
9201  TCCGGATGCC TCCGCTCGAA GTAGCGCGTC TGCTGCTCCA TACAAGCCAA
      AGGCCTACGG AGGCGAGCTT CATCGCGCAG ACGACGAGGT ATGTTCGGTT
9251  CCACGGCCTC CAGAAGAAGA TGTTGGCGAC CTCGTATTGG GAATCCCCGA
      GGTGCCGGAG GTCTTCTTCT ACAACCGCTG GAGCATAACC CTTAGGGGCT
9301  ACATCGCCTC GCTCCAGTCA ATGACCGCTG TTATGCGGCC ATTGTCCGTC
      TGTAGCGGAG CGAGGTCAGT TACTGGCGAC AATACGCCGG TAACAGGCAG   PCAM-6
9351  AGGACATTGT TGGAGCCGAA ATCCGCGTGC ACGAGGTGCC GGACTTCGGG
      TCCTGTAACA ACCTCGGCTT TAGGCGCACG TGCTCCACGG CCTGAAGCCC
9401  GCAGTCCTCG GCCCAAAGCA TCAGCTCATC GAGAGCCTGC GCGACGGACG
      CGTCAGGAGC CGGGTTTCGT AGTCGAGTAG CTCTCGGACG CGCTGCCTGC
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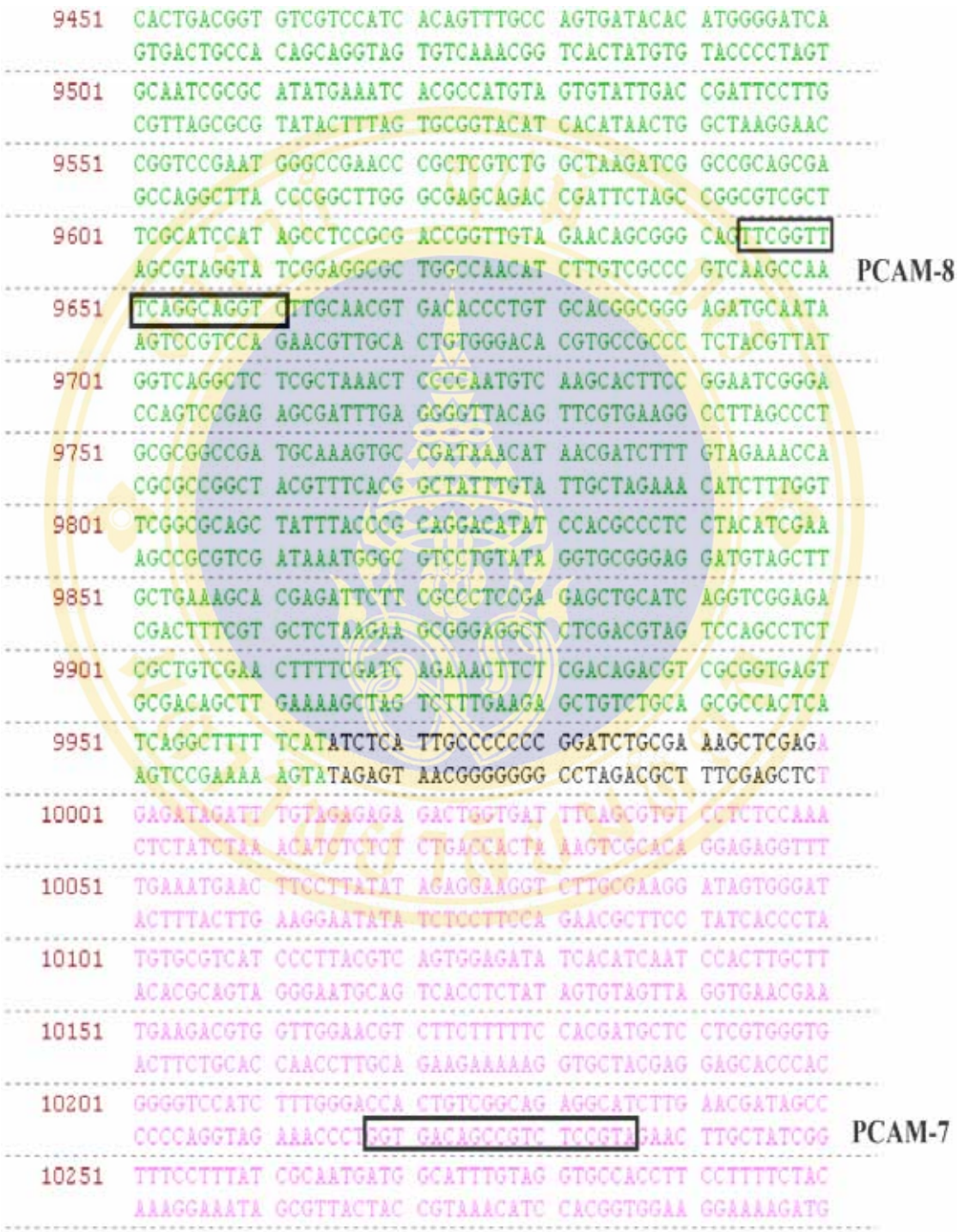

PCAM-8

PCAM-7

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10301   TGTCCTTTTG ATGAAGTGAC AGATAGCTGG GCAATGGAAT CCGAGGAGGT
        ACAGGAAAAC TACTTCACTG TCTATCGACC CGTTACCTTA GGCTCCTCCA

10351   TTCCCGATAT TACCCTTTGT TGAAAAGTCT CAATAGCCCT TTGGTCTTCT
        AAGGGCTATA ATGGGAAACA ACTTTTCAGA GTTATCGGGA AACCAGAAGA

10401   GAGACTGTAT CTTTGATATT CTTGGAGTAG ACGAGAGTGT CGTGCTCCAC
        CTCTGACATA GAAACTATAA GAACCTCATC TGCTCTCACA GCACGAGGTG

10451   CATGTTATCA CATCAATCCA CTTGCTTTGA AGACGTGGTT GGAACGTCTT
        GTACAATAGT GTAGTTAGGT GAACGAAACT TCTGCACCAA CCTTGCAGAA

10501   CTTTTTCCAC GATGCTCCTC GTGGGTGGGG GTCCATCTTT GGGACCACTG
        GAAAAAGGTG CTACGAGGAG CACCCACCCC CAGGTAGAAA CCCTGGTGAC

10551   TCGGCAGAGG CATCTTGAAC GATAGCCTTT CCTTTATCGC AATGATGGCA
        AGCCGTCTCC GTAGAACTTG CTATCGGAAA GGAAATAGCG TTACTACCGT

10601   TTTGTAGGTG CCACCTTCCT TTTCTACTGT CCTTTTGATG AAGTGACAGA
        AAACATCCAC GGTGGAAGGA AAAGATGACA GGAAAACTAC TTCACTGTCT

10651   TAGCTGGGCA ATGGAATCCG AGGAGGTTTC CCGATATTAC CCTTTGTTGA
        ATCGACCCGT TACCTTAGGC TCCTCCAAAG GGCTATAATG GGAAACAACT

10701   AAAGTCTCAA TAGCCCTTTG GTCTTCTGAG ACTGTATCTT TGATATTCTT
        TTTCAGAGTT ATCGGGAAAC CAGAAGACTC TGACATAGAA ACTATAAGAA

10751   GGAGTAGACG AGAGTGTCGT GCTCCACCAT GTTGGCAAGC TGCTCTAGCC
        CCTCATCTGC TCTCACAGCA CGAGGTGGTA CAACCGTTCG ACGAGATCGG

10801   AATACGCAAA CCGCCTCTCC CCGCGCGTTG GCCGATTCAT TAATGCAGCT
        TTATGCGTTT GGCGGAGAGG GGCGCGCAAC CGGCTAAGTA ATTACGTCGA

10851   GGCACGACAG GTTTCCCGAC TGGAAAGCGG GCAGTGAGCG CAACGCAATT
        CCGTGCTGTC CAAAGGGCTG ACCTTTCGCC CGTCACTCGC GTTGCGTTAA

10901   AATGTGAGTT AGCTCACTCA TTAGGCACCC CAGGCTTTAC ACTTTATGCT
        TTACACTCAA TCGAGTGAGT AATCCGTGGG GTCCGAAATG TGAAATACGA

10951   TCCGGCTCGT ATGTTGTGTG GAATTGTGAG CGGATAACAA TTTCACACAG
        AGGCCGAGCA TACAACACAC CTTAACACTC GCCTATTGTT AAAGTGTGTC

11001   GAAACAGCTA TGACCATGAT TACGAATTCG AGCTCGGTAC CCGGGGATCC
        CTTTGTCGAT ACTGGTACTA ATGCTTAAGC TCGAGCCATG GGCCCCTAGG

                                        HindIII
                                        ^^^^^^^^^^^^
11051   TCTAGAGTCG ACCTGCAGGC ATGCAAGCTT GGCACTGGCC GTCGTTTTAC
        AGATCTCAGC TGGACGTCCG TACGTTCGAA CCGTGACCGG CAGCAAAATG

11101   AACGTCGTGA CTGGGAAAAC CCTGGCGTTA CCCAACTTAA TCGCCTTGCA
        TTGCAGCACT GACCCTTTTG GGACCGCAAT GGGTTGAATT AGCGGAACGT
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11151  GCACATCCCC CTTTCGCCAG CTGGCGTAAT AGCGAAGAGG CCCGCACCGA
       CGTGTAGGGG GAAAGCGGTC GACCGCATTA TCGCTTCTCC GGGCGTGGCT
11201  TCGCCCTTCC CAACAGTTGC GCAGCCTGAA TGGCGAATGC TAGAGCAGCT
       AGCGGGAAGG GTTGTCAACG CGTCGGACTT ACCGCTTACG ATCTCGTCGA
11251  TGAGCTTGGA TCAGATTGTC GTTTCCCGCC TTCAGTTTAG CTTCATGGAG
       ACTCGAACCT AGTCTAACAG CAAAGGGCGG AAGTCAAATC GAAGTACCTC
11301  TCAAAGATTC AAATAGAGGA CCTAACAGAA CTCGCCGTAA AGACTGGCGA
       AGTTTCTAAG TTTATCTCCT GGATTGTCTT GAGCGGCATT TCTGACCGCT
11351  ACAGTTCATA CAGAGTCTCT TACGACTCAA TGACAAGAAG AAAATCTTCG
       TGTCAAGTAT GTCTCAGAGA ATGCTGAGTT ACTGTTCTTC TTTTAGAAGC
11401  TCAACATGGT GGAGCACGAC ACACTTGTCT ACTCCAAAAA TATCAAAGAT
       AGTTGTACCA CCTCGTGCTG TGTGAACAGA TGAGGTTTTT ATAGTTTCTA
11451  ACAGTCTCAG AAGACCAAAG GGCAATTGAG ACTTTTCAAC AAAGGGTAAT
       TGTCAGAGTC TTCTGGTTTC CCGTTAACTC TGAAAAGTTG TTTCCCATTA
11501  ATCCGGAAAC CTCCTCGGAT TCCATTGCCC AGCTATCTGT CACTTTATTG
       TAGGCCTTTG GAGGAGCCTA AGGTAACGGG TCGATAGACA GTGAAATAAC
11551  TGAAGATAGT GGAAAGGAA GGTGGCTCCT ACAAATGCCA TCATTGCGAT
       ACTTCTATCA CCTTTTCCTT CCACCGAGGA TGTTTACGGT AGTAACGCTA
11601  AAAGGAAAGG CCATCGTTGA AGATGCCTCT GCCGACAGTG GTCCCAAAGA
       TTTCCTTTCC GGTAGCAACT TCTACGGAGA CGGCTGTCAC CAGGGTTTCT
11651  TGGACCCCCA CCCACGAGGA GCATCGTGGA AAAAGAAGAC GTTCCAACCA
       ACCTGGGGGT GGGTGCTCCT CGTAGCACCT TTTTCTTCTG CAAGGTTGGT
11701  CGTCTTCAAA GCAAGTGGAT TGATGTGATA TCTCCACTGA CGTAAGGGAT
       GCAGAAGTTT CGTTCACCTA ACTACACTAT AGAGGTGACT GCATTCCCTA
11751  GACGCACAAT CCCACTATCC TTCGCAAGAC CCTTCCTCTA TATAAGGAAG
       CTGCGTGTTA GGGTGATAGG AAGCGTTCTG GGAAGGAGAT ATATTCCTTC
11801  TTCATTTCAT TTGGAGAGAA CACGGGGGAC TCTTGAC
       AAGTAAAGTA AACCTCTCTT GTGCCCCCTG AGAACTG
    1  CATGGTAGAT CTGAGGGTAA ATTTCTAGTT TTTCTCCTTC ATTTTCTTGG
       GTACCATCTA GACTCCCATT TAAAGATCAA AAAGAGGAAG TAAAAGAACC
   51  TTAGGACCCT TTTCTCTTTT TATTTTTTTG AGCTTTGATC TTTCTTTAAA
       AATCCTGGGA AAAGAGAAAA ATAAAAAAAC TCGAAACTAG AAAGAAATTT
  101  CTGATCTATT TTTTAATTGA TTGGTTATGG TGTAAATATT ACATAGCTTT
       GACTAGATAA AAAATTAACT AACCAATACC ACATTTATAA TGTATCGAAA
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151  AACTGATAAT CTGATTACTT TATTTCGTGT GTCTATGATG ATGATGATAG
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201  TTACAGAACC GACGACTCGT CCGTCCTGTA GAACGTGAAA TCAAAAAACT
     AATGTCTTGG CTGCTGAGCA GGCAGGACAT CTTGCACTTT AGTTTTTTGA
251  CGACGGCCTG TGGGCATTCA GTCTGGATCG CGAAAACTGT GGAATTGATC
     GCTGCCGGAC ACCCGTAAGT CAGACCTAGC GCTTTTGACA CCTTAACTAG
301  AGCGTTGGTG GGAAAGCGCG TTACAAGAAA GCCGGGCAAT TGCTGTGCCA
     TCGCAACCAC CCTTTCGCGC AATGTTCTTT CGGCCCGTTA ACGACACGGT
351  GGCAGTTTTA ACGATCAGTT CGCCGATGCA GATATTCGTA ATTATGCGGG
     CCGTCAAAAT TGCTAGTCAA GCGGCTACGT CTATAAGCAT TAATACGCCC
401  CAACGTCTGG TATCAGCGCG AAGTCTTTAT ACCGAAAGGT TGGGCAGGCC
     GTTGCAGACC ATAGTCGCGC TTCAGAAATA TGGCTTTCCA ACCCGTCCGG
451  AGCGTATCGT GCTGCGTTTC GATGCGGTCA CTCATTACGG CAAAGTGTGG
     TCGCATAGCA CGACGCAAAG CTACGCCAGT GAGTAATGCC GTTTCACACC   PCAM-9
501  GTCAATAATC AGGAAGTGAT GGAGCATCAG GGCGGCTATA CGCCATTTGA
     CAGTTATTAG TCCTTCACTA CCTCGTAGTC CCGCCGATAT GCGGTAAACT
551  AGCCGATGTC ACGCCGTATG TTATTGCCGG GAAAAGTGTA CGTATCACCG
     TCGGCTACAG TGCGGCATAC AATAACGGCC CTTTTCACAT GCATAGTGGC
601  TTTGTGTGAA CAACGAACTG AACTGGCAGA CTATCCCGCC GGGAATGGTG
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651  ATTACCGACG AAAACGGCAA GAAAAAGCAG TCTTACTTCC ATGATTTCTT
     TAATGGCTGC TTTTGCCGTT CTTTTTCGTC AGAATGAAGG TACTAAAGAA
701  TAACTATGCC GGAATCCATC GCAGCGTAAT GCTCTACACC ACGCCGAACA
     ATTGATACGG CCTTAGGTAG CGTCGCATTA CGAGATGTGG TGCGGCTTGT
751  CCTGGGTGGA CGATATCACC GTGGTGACGC ATGTCGCGCA AGACTGTAAC
     GGACCCACCT GCTATAGTGG CACCACTGCG TACAGCGCGT TCTGACATTG
801  CACGCGTCTG TTGACTGGCA GGTGGTGGCC AATGGTGATG TCAGCGTTGA
     GTGCGCAGAC AACTGACCGT CCACCACCGG TTACCACTAC AGTCGCAACT
851  ACTGCGTGAT GCGGATCAAC AGGTGGTTGC AACTGGACAA GGCACTAGCG
     TGACGCACTA CGCCTAGTTG TCCACCAACG TTGACCTGTT CCGTGATCGC
901  GGACTTTGCA AGTGGTGAAT CCGCACCTCT GGCAACCGGG TGAAGGTTAT
     CCTGAAACGT TCACCACTTA GGCGTGGAGA CCGTTGGCCC ACTTCCAATA
951  CTCTATGAAC TGTGCGTCAC AGCCAAAAGC CAGACAGAGT GTGATATCTA
     GAGATACTTG ACACGCAGTG TCGGTTTTCG GTCTGTCTCA CACTATAGAT
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1001  CCCGCTTCGC GTCGGCATCC GGTCAGTGGC AGTGAAGGGC GAACAGTTCC
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1051  TGATTAACCA CAAACCGTTC TACTTTACTG GCTTTGGTCG TCATGAAGAT
      ACTAATTGGT GTTTGGCAAG ATGAAATGAC CGAAACCAGC AGTACTTCTA
1101  GCGGACTTAC GTGGCAAAGG ATTCGATAAC GTGCTGATGG TGCACGACCA
      CGCCTGAATG CACCGTTTCC TAAGCTATTG CACGACTACC ACGTGCTGGT
1151  CGCATTAATG GACTGGATTG GGGCCAACTC CTACCGTACC TCGCATTACC
      GCGTAATTAC CTGACCTAAC CCCGGTTGAG GATGGCATGG AGCGTAATGG
1201  CTTACGCTGA AGAGATGCTC GACTGGGCAG ATGAACATGG CATCGTGGTG
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1251  ATTGATGAAA CTGCTGCTGT CGGCTTTAAC CTCTCTTTAG GCATTGGTTT
      TAACTACTTT GACGACGACA GCCGAAATTG GAGAGAAATC CGTAACCAAA
1301  CGAAGCGGGC AACAAGCCGA AAGAACTGTA CAGCGAAGAG GCAGTCAACG
      GCTTCGCCCG TTGTTCGGCT TTCTTGACAT GTCGCTTCTC CGTCAGTTGC
1351  GGGAAACTCA GCAAGCGCAC TTACAGGCGA TTAAAGAGCT GATAGCGCGT
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1401  GACAAAAACC ACCCAAGCGT GGTGATGTGG AGTATTGCCA ACGAACCGGA
      CTGTTTTTGG TGGGTTCGCA CCACTACACC TCATAACGGT TGCTTGGCCT
1451  TACCCGTCCG CAAGTGCACG GGAATATTTC GCCACTGGCG GAAGCAACGC
      ATGGGCAGGC GTTCACGTGC CCTTATAAAG CGGTGACCGC CTTCGTTGCG
1501  GTAAACTCGA CCCGACGCGT CCGATCACCT GCGTCAATGT AATGTTCTGC
      CATTTGAGCT GGGCTGCGCA GGCTAGTGGA CGCAGTTACA TTACAAGACG
1551  GACGCTCACA CCGATACCAT CAGCGATCTC TTTGATGTGC TGTGCCTGAA   PCAM-3
      CTGCGAGTGT GGCTATGGTA GTCGCTAGAG AAACTACACG ACACGGACTT
1601  CCGTTATTAC GGATGGTATG TCCAAAGCGG CGATTTGGAA ACGGCAGAGA
      GGCAATAATG CCTACCATAC AGGTTTCGCC GCTAAACCTT TGCCGTCTCT
1651  AGGTACTGGA AAAAGAACTT CTGGCCTGGC AGGAGAAACT GCATCAGCCG
      TCCATGACCT TTTTCTTGAA GACCGGACCG TCCTCTTTGA CGTAGTCGGC
1701  ATTATCATCA CCGAATACGG CGTGGATACG TTAGCCGGGC TGCACTCAAT
      TAATAGTAGT GGCTTATGCC GCACCTATGC AATCGGCCCG ACGTGAGTTA
1751  GTACACCGAC ATGTGGAGTG AAGAGTATCA GTGTGCATGG CTGGATATGT
      CATGTGGCTG TACACCTCAC TTCTCATAGT CACACGTACC GACCTATACA
1801  ATCACCGCGT CTTTGATCGC GTCAGCGCCG TCGTCGGTGA ACAGGTATGG
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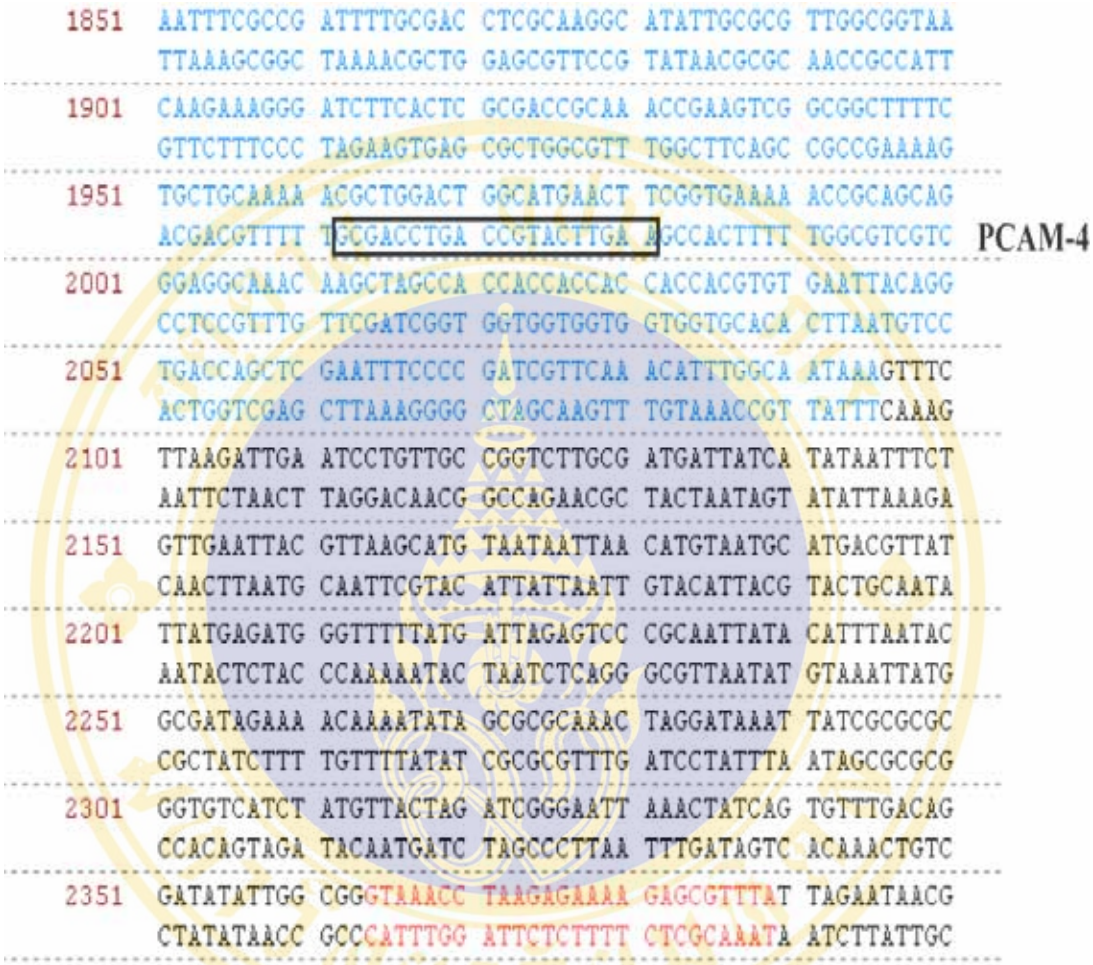

PCAM-4

APPENDIX B

1. LB medium (Luria-Bertani) (per liter)

tryptone	10	g
yeast extract	5	g
NaCl	10	g
bacto agar	15	g

2. YEP medium

bactopeptone	10	g
yeast extract	5	g
NaCl	10	g
bacto agar	15	g

3. AB medium (*Agrobacterium* culture) (per liter)

K_2HPO_4	3,000	mg
$NaH_2PO_4.2H_20$	1,000	mg
NH_4Cl	1,000	mg
KCl	150	mg
$CaCl_2.2H_2O$	12	mg
$FeSO_4.7H_2O$	2.5	mg
glucose	5,000	mg
pH 7.2		
bacto agar	15,000	mg

Autoclaving before the addition of 1M $MgSO_4.7H_2O$ 1.2 ml

4. AAM medium (per liter)

$CaCl_2.2H_2O$	440	mg
KH_2PO_4	170	mg
$MgSO_4.7H_2O$	370	mg
KCl	2.94	g

Iron

$FeSO_4.7H_2O$	28	mg
Na_2EDTA	37	mg

Micronutrients

$MnSO_4.H_2O$	16.9	mg
$ZnSO_4.7H_2O$	8.6	mg
H_3BO_3	6.2	mg
$CuSO_4.5H_2O$	25	µg
$Na_2MoO_4.2H_2O$	250	µg
$CaCl_2.6H_2O$	25	µg
KI	830	µg

Vitamins

nicotinic acid	0.5	mg
thiamine.HCl	0.5	mg
pyridoxine.HCl	0.1	mg
myo-inositol	100	mg

Amino acids

glutamine	877	mg
aspartic acid	266	mg
arginine	288	mg
glycine	75	mg
sucrose	20	mg

2,4-D	2	mg
gibberellin	0.1	mg
kinetin	0.2	mg

Adjust pH 5.8 prior to autoclaving

5. MS medium (Murashige&Skoog) (per liter)

Macronutrients

NH_4NO_3	1,650	mg
KNO_3	1,900	mg
$CaCl_2.2H_2O$	440	mg
$MgSO_4.7H_2O$	370	mg
KH_2PO_4	170	mg

Micronutrients

H_3BO_3	6.2	mg
$MnSO_4.H_2O$	6.9	mg
$ZnSO_4.H_2O$	6.14	mg
KI	0.83	mg
$Na_2MoO_4.2H_2O$	0.25	mg
$CuSO_4.6H_2O$	0.025	mg
$CoCl_2.6H_2O$	0.025	mg

Iron

$FeSO_4.7H_2O$	27.85	mg
Na_2EDTA	37.25	mg

Organic components

glycine	2.0	mg
nicotinic acid	0.5	mg
pyridoxine	0.5	mg

thiamine	0.1	mg
sucrose	30	g
pH 5.6-5.8		
gerite	3	g

6. N6D medium (callus induction) (per liter)

CHU (N6) Basal salt mixture (Sigma)	3.98	g
N_6 vitamin (x100)	10	ml
myo-inositol	100	mg
casamino acid	300	mg
proline	2.8	g
2,4-D (0.2 mg/ml)	10	ml
sucrose	30	g
pH 5.8		
gelrite	3	g
autoclaving		

N_6 vitamins (x100) 100 ml

glycine	20	mg
nicotinic acid	5	mg
pyridoxine HCl	5	mg
thiamine	20	mg

In case of selection medium, 1ml of carbenicillin (250 mg/ml) and 1 ml of hygromycin (50 mg/l) were added after autoclaving.

7. 2N6-AS medium (co-cultivation medium) (per liter)

CHU (N6) Basal salt mixture (Sigma)	3.98	g
N_6 vitamin (x100)	10	ml
myo-inositol	100	mg
casamino acid	300	mg
2,4-D (0.2 mg/ml)	10	ml
sucrose	30	g
glucose	10	g
pH 5.2		
gelrite	3	g

Autoclaving before the addition of 1 ml acetosyringone (10 mg/ml in DMSO)

8. MS-NK medium (regeneration medium) (per liter)

MS salts (Sigma)	4.33	g
MS organics (x1000)	1	ml
myo-inositol	100	mg
casamino acid	2	g
1-naphthaleneacetic acid (NAA)	0.2	mg
kinetin	2	mg
sucrose	30	g
sorbitol	30	g
pH 5.8		
gelrite	3	g

Autoclaving before the addition of 1 ml carbenicillin (250 mg/ml) and 1 ml hygromycin (50 mg/ml)

9. MS-HF medium (root induction medium) (per liter)

MS salts (Sigma)	4.33	g
MS organics (x1000)	1	ml
myo-inositol	100	mg
sucrose	30	g
pH 5.8		
gelrite	3	g

Autoclaving before the addition of 1 ml carbenicillin (250 mg/ml) and 1 ml hygromycin (50 mg/ml)

10. RMOP medium (tobacco transformation medium) (per liter)

MS salts (Sigma)	4.33	g
N^6-benzyladenine (BA)	1	mg
1-naphthaleneacetic acid (NAA)	0.1	mg
thiamine	1	mg
inositol	100	mg
sucrose	30	g
gelrite	3	g
pH 5.8		

RESEARCH GRANTS

1. Plant Biochemistry and Molecular Genetics Laboratory, Department of Biotechnology, Faculty of Science, Mahidol University

2. Partially supported by Ministry of Education, Commission of Higher Education, subproject “Graduate Study and Research in Agricultural Biotechnology” (2001-2003)

3. Partially supported by the Thesis Grant, Faculty of Graduate Studies, Mahidol University (2003)

AWARDS

1. The Outstanding Student Award from The Professor Dr.Tab Nilanidhi Foundation (The Tab foundation), Faculty of Science, Chulalongkorn University (2001)

2. Outstanding presentation

Yookongkaew N., Ngampanya B., Narangajavana J. Transgene integration and promoter methylation induce gene silencing in transgenic rice. In: The Agbiotech Graduate Conference I, Bangkok, Thailand (2004)

EXPERIENCES

1. Workshop on “Application of PCR technique”, July 28-30, Department of Biotechnology, Faculty of Engineering and Industrial Technology, Silpakorn University Nakornpathom, Thailand (2003)

2. Teaching Assistant in SCBT 407 Plant Biotechnology Laboratory Course, Department of Biotechnology, Faculty of Science, Mahidol University (2003)

3. Workshop on “DNA Microarray Data Analysis”, August 23-27, National Center of Genetic Engineering and Biotechnology (BIOTECH), Thailand Science Park, Pathumthani, Thailand (2004)

PRESENTATIONS

Oral presentation

1. Yookongkaew N., Ngampanya B., Narangajavana J. Transgene integration patterns and DNA methylation associated with gene silencing in transgenic rice. In: The 1st International Conference on Rice for the Future, August 31- September 3, Kasetsart University, Bangkok, Thailand (2004)

Poster presentations

1. Yookongkaew N., Ngampanya B., Narangajavana J. Differential expression and silencing of transgene in transgenic rice correlated with degree of promoter methylation. In: Proceeding of the 15th Annual Meeting of Thai Society for Biotechnology, February 3-6, Chiang Mai, Thailand (2004)

2. Yookongkaew N., Ngampanya B., Narangajavana J. Transgene integration and promoter methylation in transgenic rice. In: The AgBiotech Graduate Conference I, March 18-19, Bangkok, Thailand (2004)

3. Yookongkaew N. Chomchan P., Narangajavana J. Differential expression of transgene in transgenic tobacco obtained from micropropagation correlated with degree of DNA methylation. In: The 5th Princess Chulaporn International Science Congress, August 16-20, Bangkok, Thailand (2004)

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