FUNCTIONAL STUDY OF STARCH BIOSYNTHESIS RELATED GENE USING CASSAVA GENETIC TRANSFORMATION SYSTEM

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ABSTRACT

Cassava (*Manihot esculenta* Crantz) is a tropical crop grown for its starchy thickened roots. Cassava starch is often modified to obtain products that meet the criteria for numerous applications. Genetically modified cassava might offer an alternative way without having to go through environmentally unfriendly treatment during chemical modification of native starch. The establishment of a transformation system for cassava also allows plant scientists to study the function of genes in cassava. In this study, a protocol for somatic embryogenesis and organogenesis of the two cassava cultivars, Kasetsart 50 (KU50) and Hanatee, were optimized. A system for cyclic somatic embryogenesis induction was carried out to produce young green cotyledons for transformation experiments. Suppression of the endogenous granule-bound starch synthase I (GBSSI) gene in cassava was carried out using RNA interference (RNAi) technique. Molecular analysis of the transgenic cassava indicated the presence of multiple copies of GBSSI-RNAi transgene in the transgenic cassava genome, which correlated with a dramatic decrease in GBSSI transcript level. By subjecting the *in vitro* storage roots of the transgenic cassava to iodine staining, the results further confirmed that suppression of the cassava endogenous GBSSI gene by RNAi negatively affected the amylose content of the roots. In addition to cassava, transformation of potato and tobacco were carried out to study the effect of the cassava phloem-specific promoter p54. Unfortunately, the cassava promoter could not be used to drive expression of foreign genes in these model plants. Due to the availability of an efficient transformation system and the ability of potato plants to form *in vitro* mini-tubers, this system would be most suitable to use in comparative analysis of the starch biosynthesis related genes from cassava.

KEY WORDS: CASSAVA/ GENETIC TRANSFORMATION/ RNA INTERFEREANCE/ GRANULE BOUND STARCH SYNTHASE

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การศึกษาหน้าที่ของยีนในกระบวนการสังเคราะห์แป้งของมันสำปะหลังโดยวิธีการถ่ายยีน
(FUNCTIONAL STUDY OF STARCH BIOSYNTHESIS RELATED GENE USING CASSAVA GENETIC TRANSFORMATION SYSTEM)

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บทคัดย่อ

มันสำปะหลังเป็นพืชเศรษฐกิจที่มีความสำคัญของประเทศไทย สืบเนื่องจากข้อจำกัดในเรื่องของคุณสมบัติของแป้งมันสำปะหลัง การนำแป้งมันสำปะหลังไปใช้เป็นวัตถุดิบในอุตสาหกรรมต่างๆจึงยังไม่แพร่หลายเท่าที่ควร ในปัจจุบัน เทคโนโลยีชีวภาพได้เข้ามามีบทบาทที่สำคัญในการพัฒนาเพื่อให้ได้สายพันธุ์พืชที่มีคุณสมบัติตามต้องการ นอกจากนี้ยังสามารถนำมาใช้เป็นเครื่องมือในการศึกษาหน้าที่ของยีนในกระบวนการต่างๆของพืชโดยการศึกษาผลกระทบที่เกิดขึ้นเมื่อพืชมีการแสดงออกของยีนดังกล่าวในระดับที่ต่างออกไปจากเดิม งานวิจัยนี้จึงมุ่งเน้นการพัฒนาระบบการถ่ายยีนในมันสำปะหลังสายพันธุ์ไทยจำนวน 2 สายพันธุ์ได้แก่พันธุ์เกษตรศาสตร์ 50 และพันธุ์ห้านาที โดยมีวัตถุประสงค์เพื่อรองรับการศึกษาหน้าที่ของยีนเพื่อนำไปสู่การพัฒนาปรับปรุงเพื่อให้ได้สายพันธุ์มันสำปะหลังที่มีคุณสมบัติตามต้องการต่อไป งานวิจัยนี้ได้ทำการชักนำให้เกิด somatic embryogenesis เพื่อให้ได้ cotyledon ที่นำไปใช้ในการถ่ายยีน การทดสอบความต้านทานต่อยาปฏิชีวนะเพื่อใช้คัดเลือกเนื้อเยื่อที่ได้รับการถ่ายยีน การชักนำให้เกิดรากสะสมภายในสภาวะปิดเพื่อใช้ศึกษาลักษณะของแป้งที่เปลี่ยนไป จากการยับยั้งการแสดงออกของยีน granule-bound starch synthase I (GBSSI) ของมันสำปะหลังด้วยวิธี RNA interference (RNAi) พบว่าต้นที่ได้รับการถ่ายยีนมีการแสดงออกของยีน GBSSI ลดลง ส่งผลให้มีการสังเคราะห์ amylose ในเนื้อเยื่อรากสะสมในปริมาณที่ลดลง นอกจากการถ่ายยีนเข้าสู่มันสำปะหลัง งานวิจัยนี้ยังได้ทำการถ่ายยีนเข้าสู่มันฝรั่งและยาสูบ โดยมีวัตถุประสงค์ที่จะศึกษาบทบาทของโปรโมเตอร์ p54 ซึ่งเป็นโปรโมเตอร์ที่คัดแยกมาจากมัน สำปะหลังและสามารถควบคุมให้เกิดการแสดงออกของยีนเฉพาะในเนื้อเยื่อท่อลำเลียงเท่านั้น แต่จากผลการทดลองนี้พบว่าโปรโมเตอร์ p54 ไม่สามารถชักนำให้เกิดการแสดงออกของยีนในพืชต้นแบบทั้งสองชนิดได้เลย อย่างไรก็ตาม การทดลองนี้ได้ชี้ให้เห็นถึงประโยชน์ของการนำมันฝรั่งมาใช้เป็นต้นแบบในการศึกษาหน้าที่ของยีนในกลไกการสร้างแป้งควบคู่ไปกับระบบที่ศึกษาในมันสำปะหลังโดยตรง เนื่องจากระบบการถ่ายยีนในมันฝรั่งเป็นระบบที่มีประสิทธิภาพสูง อีกทั้งยังสามารถตรวจสอบคุณภาพแป้งในหัวมันฝรั่งจิ๋วได้อย่างชัดเจนอีกด้วย

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LIST OF ABBREVIATION

ATP	adenosine triphosphate
bp	base pair
BA	N^6-benzyladenine
ºC	degree Celsius
cm	centrimeter
cv.	cultivar
dNTP	deoxynucleoside-5'-triphosphate
DEPC	diethyl pyrocarbonate
DNA	deoxy ribonucleic acid
cDNA	complementary deoxy ribonucleic acid
E. coli	*Escherichia coli*
EDTA	disodium ethylene diaminetetraacetic acid
et al.	et. Alli (Latin), and others
hr	hour
hpt, hyg^r	hygromycin resistance gene
g	gram
g	gravity
gfp	green fluorescent protein gene
GFP	green fluorescent protein
gus	β-glucuronidase gene
GUS	β-glucuronidase
kb	kilobase pair
kDa	kilodalton
l	liter
LB	left border
LB	Luria-Bertani medium

LIST OF ABBREVIATION (continued)

M	molar
MAT	Multi-Auto-Transformation
µM	micromolar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mRNA	messenger ribonucleic acid
MS	Murashige and Skoog medium
MW	molecular weight
NAA	1-naphthalene acetic acid
ng	nanogram
OD	optical density
PCR	polymerase chain reaction
psi	pounds per square inch
RB	right border
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolution per minute
RT-PCR	reverse-transcription-polymerase chain reaction
sec	second
TBE	Tris-boric/EDTA buffer
Tris-HCl	Tris-hydrochloric acid
UV	ultra violet
V	volts
X-gluc	5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid

CHAPTER I
INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a tropical crop grown for its starchy thickened roots mainly by peasant farmers, for whom it is a staple food. Due to its tolerance to adverse environmental conditions and flexible harvesting time, cassava has played an important role underpinning food security in many developing countries. In term of nutritional value, cassava provides a cheap source of dietary carbohydrate energy (720.1 x 1,012 kJ day^{-1}) to over 600 million people in the tropics, ranking fourth after rice, sugar and maize and sixth among crops in global production (Baguma *et al.*, 2003). However, the low protein content and high cyanogenic compounds of cassava has made it unsuitable for human and animal consumption.

In Thailand, traditional breeding has successfully been utilized to obtain new cassava cultivars. However, this is still very difficult to achieve since the allopolyploid cassava plants show high heterozygosity and low natural fertility. To overcome these problems, transgene technology could be adopted to introduce single or even quantitative traits without the problems of linkage encountered in traditional breeding and to extend the genetic pool of useful genes beyond the species (Taylor *et al.*, 2004). The areas where genetic engineering can have an impact on cassava include yield enhancement, nutritional improvement, pest and disease resistance, and also production of novel compounds for value-added products. By combining traditional breeding and genetic engineering, novel cassava cultivars and cassava-derived products could be produced. Compared with other plant species, however, the lack of research and funding in cassava biotechnology has become the major factor that limits the utilization of cassava in both food and non-food industries.

To date, major advancements in cassava biotechnology have been made by International Center for Tropical Agriculture (CIAT) with the aims to eliminate viral infection, and to promote vegetative propagation, conservation and exchange of cassava germplasms (Roca, 1984). With the main objective to improve the livelihood of people living in Africa and Latin America, the researches at CIAT therefore focus on the cassava cultivars that are mainly grown in those areas. For Thai cassava cultivars; Kasetsart 50 (KU50) and Hanatee, protocols for somatic embryogenesis and organogenesis have been established (Saelim *et al.*, 2006). These protocols were then followed throughout this study.

A prerequisite for genetic engineering of cassava is a reliable transformation system. Since 1996, there are various reports on cassava transformation, which utilized *Agrobacterium* and biolistic bombardment to transform the cassava embryogenic suspension culture (Raemakers *et al.*, 2001; Schopke *et al.*, 1996) and somatic cotyledons from which plants were regenerated via organogenesis (Li *et al.*, 1996) or embryogenesis (Sarria *et al.*, 1996). To date, continuous improvement of cassava transformation and regeneration systems has still been made (Gonzalez *et al.*, 1998; Sarria *et al.*, 2000; Zhang *et al.*, 2000; Zhang & Puonti-Kaerlas, 2000). However, there is still no report on genetic transformation of Thai cassava cultivars probably due to the lack of an efficient transformation system in these cultivars.

In targeted gene expression experiments, a tissue-specific promoter is required. At present, there are only a few promoters available for cassava; unfortunately, none of which is a root-specific promoter. In this study, a promoter of c54 gene, which encodes a glutamic-acid rich protein in cassava, would be used to transform potato and tobacco. According to Zhang *et al*. (2003a), this promoter, namely p54, is predominantly active in vascular tissues of both cassava and *Arabidopsis*. If the p54 could behave in potato the same way it does in cassava, comparative analysis of the cassava genes could be carried out in potato, in which an efficient transformation system was already established. Along with the study of the cassava root-specific promoter p54, the effect of RNA interference in suppression of endogenous expression of a cassava gene was investigated. By transforming cassava with DNA fragment containing only the gene of interest, the transgenic lines obtained from this study would lack not only ‘backbone’ sequence of the vector but also an antibiotic

resistance gene. These marker-free transgenic lines should extremely benefit further utilization of transgenic cassava, if they would be released for commercial production in the future.

The objectives of this research could be summarized as follows:

1. Suppression of a starch biosynthetic gene, namely granule bound starch synthase I (GBSSI), in cassava using RNA interference (RNAi) technique.
2. Investigation whether the cassava tissue-specific promoter p54 could be used to drive expression of foreign genes in potato.

CHAPTER II

LITERATURE REVIEWS

1. Cassava

Cassava (*Manihot esculenta* Crantz), also called manioc, tapioca or yucca, is one of the most important food crops in tropical and subtropical countries. Cassava is native to South America and was domesticated less than 10,000 years ago. Early European sailors recognized the advantages of the crop and carried it to Africa. From there, traders later introduced it to Asia. Cassava is a woody perennial shrub or a small tree with finger-like leaves. Its tuberous roots contain starch up to 85% of their dry weight and serve as a staple food for over 600 million people worldwide (Cock, 1985). Cassava ranks second to sugarcane in terms of the most efficient producer of carbohydrate under optimal growing conditions. Compared to other major food crops, cassava is highly drought tolerance and has low demand for nutrients. Therefore, acceptable yield of cassava could be obtained even under marginal environmental conditions (Cock, 1985). Cassava is Africa's second most important food crop, after maize in terms of calories consumed. In the early 1960s, cassava production in Africa accounted for 42% of the global production. Thirty years later, in the early 1990s, Africa produced half of the world cassava output, primarily because Nigeria and Ghana increased their production four fold. As a result, utilization of cassava and cassava-derived products has been increased. In Latin American, the Consortium of Latin America and the Caribbean for cassava research for development (CLAYUCA), a private-public initiative, has promoted the utilization of cassava in animal feed, ethanol production and starch industries (Ceballos, 2002). In South East Asia, cassava is currently being exploited as an alternative source of fuel for automobile (Sriroth *et al.*, 2003).

In the northeastern and eastern regions of Thailand, cassava is usually grown by smallholders in upland areas on poor soils with low or unpredictable amount of rainfall. Since most cassava farmers are poor, they do not apply sufficient fertilizer to the plants and this can lead to a decline in soil fertility, which in turn exacerbates low yields. A research by Kasetsart University, Thailand, has shown that cassava cultivation may cause twice much soil erosion as the cultivation of mung bean, and three times as much as that caused by maize, sorghum and peanut (Puttacharoen *et al.*, 1998). For Thailand, rice is a staple food, while cassava has traditionally been considered as a snack food or as an ingredient used in desserts. Since the 1960s, cassava roots have been used for production of dry chips and pellets, mainly for export. Presently, more and more cassava roots are being utilized for the production of starch. About 40% of cassava starch is used domestically, mainly as chemically or physically modified starch. The remaining 60% is exported, both in the form of native and modified starch. The export of Thai cassava starch and starch-derived products has increased dramatically, especially during the past ten years (Thai Tapioca Flour Industries Trade Association, 1994). To expand the use of cassava starch, a lot of research is being conducted with the aim to convert starch to other value-added products. In Thailand, cassava breeding is under the responsibility of Rayong Field Crops Research Center (Rayong-FCRC), Department of Agriculture (DOA), as well as of Kasetsart University (KU). The goal of cassava breeding in Thailand is to increase yield, both in terms of root yield and root dry matter content, and to improve diversification of genetic base, resistance to pests and diseases, as well as adaptability to a wide range of growing conditions. Other considered criteria include early harvest time, stake quality and desirable root shape (Sriroth *et al.*, 2002)

Cassava starch has long been utilized in various industries, for instance, textile and paper-coating industries. In the adhesive-making industry, cassava starch is often preferred since it yields the products that are highly viscous and stable at neutral pH. In food industry, cassava starch is widely utilized. This mainly results from its remarkable characteristics, including colorless, odorless and very bland in flavor, thus making it extremely convenient when blended with other flavoring and color agents. However, there is a major drawback in using cassava in food products. When heated in water, cassava starch is gelatinized and becomes fluid upon cooling. Therefore, it

can not be used to make jellies or pudding. Since the cassava starch solution is highly stable, it can not be separated into an insoluble form, as in the case with corn and potato starch solution ("retrogradation"). To solve this problem, cassava starch must be modified to obtain products that meet the criteria for their applications.

Cassava starch consists, as do other plant starches, of two glucan polymers: amylose and amylopectin. In general, the amylose content of cassava starch ranges from 14 to 24% (Asaoka *et al.*, 1991; Defloor *et al.*, 1998; Moorthy & Ramanujan, 1986). With the high genetic diversity of cassava plant and/or with the use of genetic engineering, amylose-free cassava cultivars have been identified (Raemakers *et al*., 2005; Ceballos *et al*., 2007). It was found that the amylose-free cassava starch differed significantly from amylose-containing cassava starch, and from other amylose-containing and amylose-free starches (Raemakers *et al.*, 2005).

2. *In vitro* plant regeneration

A success in plant transformation experiment basically requires two integral components; a high efficiency transformation method and an efficient tissue culture and plant regeneration system. At present, various methods for plant regeneration are currently available. Some plant species may be amenable to regeneration by a variety of methods, while some may respond to only one particular protocol. However, some protocols, at least at the moment, are clearly more efficient than others. For example, regeneration from immature embryo-derived somatic embryos is the favored method for monocot species. According to Figure 2.1, an overview of the cassava tissue culture is showed. This also includes various regeneration systems developed using immature leaf lobes or meristem. Somatic embryogenesis is the most routinely used method of *de novo* plant regeneration of cassava *in vitro*. Continuous culture of somatic embryos on auxin supplemented medium allows a cyclic system of somatic embryogenesis. Such cultures can be maintained as continuous explant source for plant regeneration and transformation studies. FEC can be used to establish embryogenic suspension cultures. This callus could either be cultured for maturation or for further proliferation.

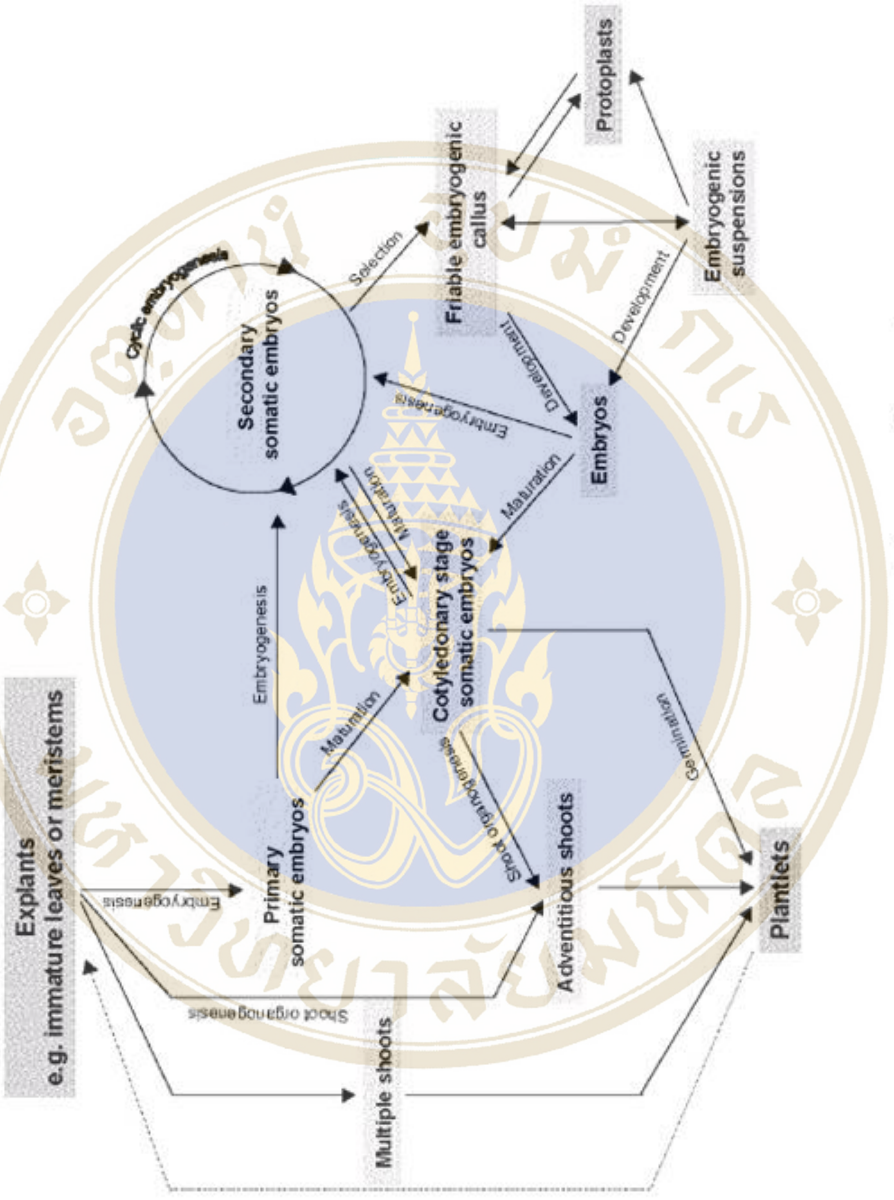


Figure 2.1 Overview of cassava tissue culture (Zhang *et al.*, 2001)

2.1 Somatic embryogenesis

Somatic embryogenesis is an asexual form of plant propagation that mimics many of the events of sexual reproduction. In somatic embryogenesis, embryo-like structures are formed from somatic tissues composing of single cell or small group of cells (Figure 2.2). The embryos will further develop to form globular-, heart- and torpedo-stage somatic embryos, from which plants can be regenerated. In the globular stage, the embryo develops radial patterning through a series of cell divisions. The outer layer of cells differentiates to the 'protoderm', while the inner layer is composed of two cell types, each of which has distinct developmental fates. Cells in the apical layer will give rise to cotyledon and shoot meristem, while those in the lower layer produce hypocotyl and root meristem. For heart-stage embryos, bilateral symmetry is apparent. In the subsequent torpedo and cotyledonary stages of embryogenesis, the embryo completes its growth by elongating and enlarging. These somatic embryos can be produced either directly or indirectly. In direct somatic embryogenesis, the embryo is formed from a cell or small group of cells without having to go through callus stage. This process is commonly observed in some tissues, particularly reproductive tissues such as nucellus, styles or pollen. However, it is generally rare in comparison with the indirect somatic embryogenesis. In indirect somatic embryogenesis, callus is first produced from explants. Embryos can then be produced from the callus tissue or from a cell suspension produced from that callus (Sharp *et al.*, 1980).

The *in vitro* somatic embryogenesis generally proceeds in two distinct stages. The initial stage, which involves embryo initiation, is totally dependent on a high concentration of auxin, while the second stage requires no or very low level of auxin. The somatic embryogenesis could be improved by supplying a source of reduced nitrogen, such as specific amino acids or casein hydrolysate (Hita *et al.*, 2003; Leljak-Levanic *et al.*, 2004). Furthermore, silver nitrate has been shown to be effective in improving somatic embryogenesis and plant regeneration in a number of crop species, including cassava (Zhang *et al.*, 2001). The Ag^{+} ion can prevent a wide variety of plant responses by inhibiting the physiological action of ethylene, a potential inhibitor of many plant regeneration systems (Kong & Yeung, 1994).

To date, various protocols for somatic embryogenesis of cassava have been reported (Schopke *et al.*, 1996; Stamp & Henshaw, 1982, 1987; Szabados *et al.*, 1987). In terms of the type of medium, half- and full-strength MS media gave no significant difference in the production efficiency of cassava somatic embryos (Groll *et al.*, 2002). To induce somatic embryogenesis in cassava, a two-step procedure is generally followed. The first stage involves the induction of somatic embryos on medium containing 2,4-D. Subsequently, the embryos are transferred to medium with 2,4-D, BA and with or without GA to promote shoot development and adventitious rooting (Stamp & Henshaw, 1987; Szabados *et al.*, 1987). Unfortunately, the development observed in the second stage is often limited to apical shoot elongation with no growth of the root axis. To induce adventitious root formation, the embryos exhibiting shoot elongation need to be excised and subcultured onto rooting medium.

2.2 Organogenesis

While somatic embryogenesis relies on plant regeneration through a process analogous to zygotic embryo germination, organogenesis relies on the production of organs, either directly from an explant or from a callus culture. There are three methods of plant regeneration via organogenesis. The first two methods depend on adventitious organs arising either from a callus culture or directly from an explant, while the last method requires the formation of axillary buds from some types of tissue culture to regenerate to whole plant (Flick *et al.*, 1983). Organogenesis relies on the inherent plasticity of plant tissues, and is regulated by altering the components in plant cell culture medium. In particular, it is the auxin to cytokinin ratio that determines which developmental pathway the regenerating tissue will take. To induce shoot formation, a high cytokinin to auxin ratio must be used (Sugiyama, 1999). For cassava, shoot organogenesis from somatic cotyledons is a rapid regeneration system with minimal somaclonal variation (Li *et al.*, 1998). In addition, it has been demonstrated to be compatible with gene transfer via particle bombardment (Zhang *et al.*, 2000) and *Agrobacterium*-mediated transformation (Li *et al.*, 1996; Puonti-Kaerlas *et al.*, 1997).

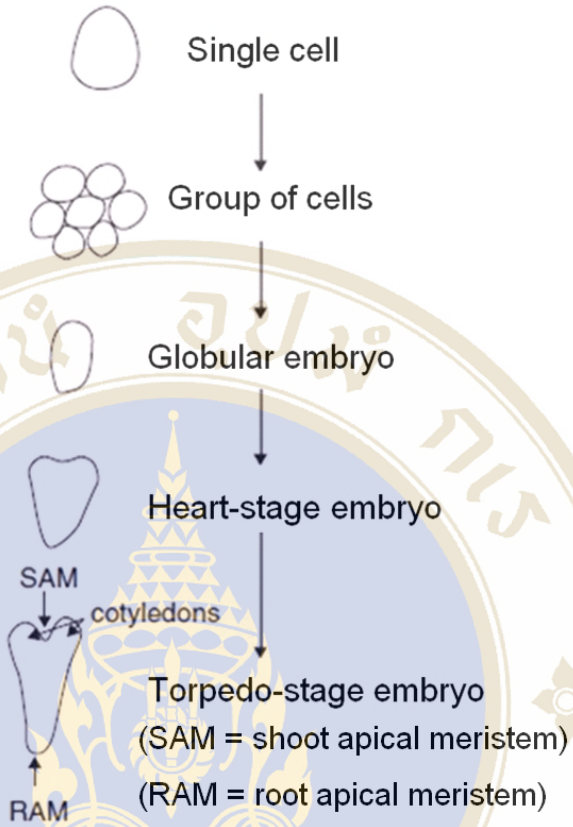


Figure 2.2 Sequential stages of somatic embryo development

Various developmental stages of somatic embryos, including globular, heart and torpedo, could be generated from single cells or a small group of cells. These sequential stages of the somatic embryo are similar to those observed during zygotic embryo development (www.oup.com/uk/orc/bin/0199254680/ch02.pdf).

3. Gene transfer into plants

Transgene expression in plants is highly variable, even among plants that have been transformed with the same construct. Many factors may be responsible for variable transgene expression, including the tendency for exogenous DNA to undergo rearrangement prior to integration, position effects, the effects of transgene copy number, and the effects of DNA methylation. It is therefore important to learn as much as possible about the mechanisms of transgene integration, and how the structure and organization of transgene affect its stability and expression level. Since these could be affected by the method that is used to deliver transgene into plant cells, detailed information of the two methods used in plant transformation, *Agrobacterium*- and biolistic-mediated transformation, was described.

3.1 *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation has widely been used to induce foreign DNA into dicotylydonous plants. This system is based on the natural process of tumor formation by *Agrobacterium tumefaciens*. This bacterium is capable to transfer a defined region of DNA (T-DNA) from the tumor-inducing (Ti) plasmid into plant cells using a process thought to be analogous to bacterial conjugation (Figure 2.3). To induce for T-DNA integration into plant genome, two essential components encoded on both Ti plasmid and/or bacterial choromosome are required. These include the *cis*-acting T-DNA border sequences and the *trans*-acting virulence (*vir*) genes. The right border sequence was observed to be extremely essential for polarity of the T-DNA transfer (Wang *et al.*, 1984). This was based on the observation that deletion of the left border did not significantly affect the T-DNA transfer, while deletion of the right border abolished tumorigenicity of the *Agrobacterium*. During the T-DNA transfer, the products of *virD1* and *virD2* genes catalyze single-strand nicks and double-strand breaks at the T-DNA border sequences of *Agrobacterium*. Upon direct interaction between the right border and *virC1* protein, the T-DNA transfer complex is formed. Stability of the T-DNA complex could be enhanced by binding with *virE2* protein, a single-stranded DNA-binding protein. Additionally, the protein component of the

protein-T-DNA complex also facilitates the recognition of membrane pores that provide the exit of the T-DNA intermediates. The products of the *virB* operon have been proposed to form a membrane-localized complex that mediates the passage of T-DNA intermediates through the bacterial membrane. After entering plant cells, the T-DNA transfer complex is directed to the plant nucleus. This process is mediated by the eukaryotic (plant) nuclear-targeting signal carried by the N-terminal region of the *virD2* protein. Although it is unknown whether the T-DNA is integrated into the plant genome by single- or double-stranded DNA intermediates, genetic-mapping studies indicate that the T-DNA is inserted into plant genome at random. During the T-DNA integration, deletion, inversion and duplication of the target plant DNA sequences can often be found, thus suggesting that T-DNA integration may depend on the endogenous recombination system in plants. According to Somers *et al.* (2004), T-DNA integration is carried out via illegitimate recombination, which occurs between DNA sequences that contain only a few identical nucleotides, followed by small deletions in the genomic integration site.

The most commonly used plant transformation vectors are binary vectors, whose name indicates their ability to replicate in both *E. coli* and *A. tumefaciens*. The presence of origin of replication and selectable markers that are functional in both *E. coli* and *A. tumefaciens* allows plant researchers to easily transfer the binary vectors from *E. coli* to *A. tumefaciens* by means of conjugation, using a helper plasmid for mobilization, or by direct transfer via transformation or electroporation. Furthermore, multiple restriction sites located between the two border sequences of the binary vectors also facilitate cloning of foreign DNA and a dominant selectable marker gene fused with a plant promoter. Since the right border sequence is essential for T-DNA transfer, it is desirable that foreign DNA is located next to the right border sequence. Otherwise, it would be possible to obtain transgenic plants that contain only the selectable marker gene. At present, most binary vectors that are commercially available are rather large and carry a limited number of cloning sites.

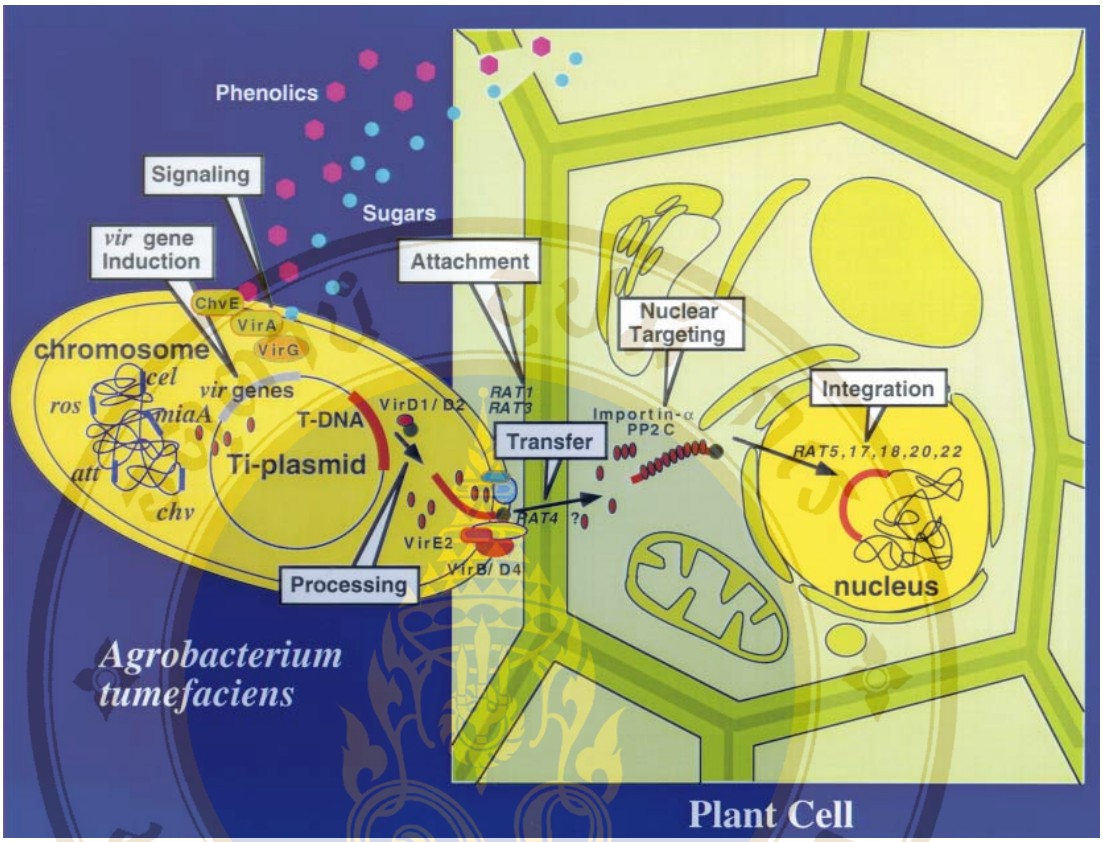


Figure 2.3 Schematic diagram of the *Agrobacterium* infection process

Critical steps that occur to or within the bacterium (chemical signaling, *vir* gene induction, and T-DNA processing) and within the plant cell (bacterial attachment, T-DNA transfer, nuclear targeting, and T-DNA integration) along with genes and/or proteins known to mediate these events were specified (Gelvin, 2000).

To date, *Agrobacterium*-mediated gene delivery has routinely been applied to various dicotyledonous crops including cassava (Li *et al.*, 1996; Schopke *et al.*, 1996). Since the end of 1980s, *Agrobacterium*-mediated transformation of monocot plants has also been reported (Creissen *et al.*, 1989; Enriquez-Obregon *et al.*, 1989; Machida *et al.*, 1989).

3.2 Biolistic-mediated transformation

Particle bombardment, or biolistics, is commonly used for genetic transformation of plants. Millions of DNA-coated metal particles are shot at target cells or tissues using a biolistic device or gene gun. The DNA eluted off the particles that lodge inside the cells may be stably incorporated into the host chromosomes. This procedure has been applied to a wide range of plant species, including those that have been recalcitrant to transformation by any other procedures. The earliest use of microprojectiles to facilitate gene transfer into plants was reported by MacKenzie *et al.* (1966). Subsequently, Finer & McMullen (1991) further improved the system and constructed the Particle Inflow Gun (PIG), which is inexpensive and simple to use. As a result, biolistics has become the second most widely used technique for plant transformation after *Agrobacterium-mediated* transformation. This technique has successfully been used to transform meristems and tissues with high regeneration potential with no limitation of *Agrobacterium*-related host specificity and tissue culture-related regeneration difficulties. In addition, it has also been applied to transform organelles such as chloroplasts (Svab *et al.*, 1990).

Transgene integration in particle bombardment is more complicated than that in *Agrobacterium*-mediated transformation. It is because the DNA introduced by bombardment does not need to contain particular sequence motifs as in *Agrobacterium*-mediated transformation. Compared with *Agrobacterium* transformation, only a few equivalent studies have been carried out to investigate the transgene integration events in particle bombardment. Based on the results obtained from co-transformation of rice with up to 13 plasmid vector, Chen *et al.* (1998) indicated that there was no preference for the integration of particular transgenes and the insertion mechanisms operated independently of gene sequence. According to

fluorescence *in situ* hybridization (FISH) analysis of transgenic oat, transgene integration was shown to randomly occur and there was no preference for particular regions in the genome (Svitashev *et al.*, 2000). However, the integration events often occur at the telomeric and sub-telomeric regions, which are typically gene rich.

In particle bombardment, two-phase integration mechanism requires multiple-tier organization, in which clusters of transgene are joined end-to-end with interspersed regions of genomic DNA (Kohli *et al.* 1998). In the first phase, exogenous DNA becomes ligated together to form transgene arrays lacking genomic DNA. These arrays are the substrates for integration, presumably by interacting with randomly-occurring breaks in the endogenous chromosomes. Theoretically, this process could evenly occur throughout the genome, thus resulting in many unlinked transgenic loci. In fact, it has been observed that additional transgene arrays tend to integrate at the same locus. The reason for such clustering may reflect the recruitment of DNA repair complexes to the original site of integration caused by the introduction of many local double strand DNA breaks. Strand exchange and recombination at such loci could then result in the elimination of variable-length regions of host DNA at the integration site, a common characteristic observed in transgenic plants. In some cases, the clustered transgene arrays may be interspersed with an extremely large region of genomic DNA (Kohli *et al.*, 1998). This may reflect localized damage, caused by the metal particles, in a specific region of the nucleus, where tertiary structure brings looped DNA strands into close physical proximity.

Integration of transgene into plant cells is proposed to mimic a wound response process in a way that it includes the induction of DNA repair enzymes such as nucleases and ligases. The presence of these enzymes and an excess of foreign DNA would cause several copies of the transgene to link together to form concatemers. Additionally, concatemerisation might be stimulated by homology present in the transforming plasmids, particularly in co-transformation experiments in which various different constructs carry the same ‘backbone’ plasmid. However, co-transformation and co-integration were shown to occur only when two non-homologous minimal cassettes were used for transformation (Fu *et al.*, 2004). Therefore, such homology might not be as important as the presence of the free DNA ends.

4. Genetic markers in plant transformation

Generally, successful genetic transformation requires not only a good gene delivery protocol but also an efficient selection system. The most frequently used marker genes for plant transformation are those encoding antibiotic or herbicide resistance (Table 2.1). However, these marker genes pose various concerns in public mind, particularly having the marker gene left in the final product of plant transformation. The presence of marker gene may result in the toxicity or allergenicity caused by the gene product and may have an impact on horizontal gene transfer from transgenic plant to wild relatives or to pathogenic bacteria (Doerfler *et al.*, 1997; Schubbert *et al.*, 1994).

In plants, the most extensively used marker gene is neomycin phosphotransferase II (*nptII*) gene from the bacterial transposon Tn5, which confers resistance to kanamycin. Additionally, hygromycin phosphotransferase (*hpt*) gene from *E. coli* has also been used as selectable marker in several plant species (Waldron *et al.*, 1985). Other selectable markers include bleomycin resistance gene (Hille *et al.*, 1986) and dihydrofolate reductase gene.

In addition to resistance to antibiotics, various types of negative selection markers that are lethal or interfere with normal development of plants have been developed. An example of this type of marker is the *tms2* gene of *A. tumefaciens* (Karlin-Neumann *et al.*, 1991). The *tms2* gene encodes enzyme that catalyzes the conversion of indole-3-acetamide (IAM) to indole-3-acetic acid (IAA), a natural plant auxin. At low concentrations, auxins generally act as regulators of plant growth and development. However, they are extremely toxic to plant cells when presence at elevated concentrations. To be able to use *tms2* gene as selectable marker in plant transformation, the type of promoter that will be used to drive expression of the *tms2* gene will have to be chosen more carefully.

Table 2.1 Selectable marker genes used in plant transformation (Zhang *et al.*, 2000)

Marker genes	Enzyme encoded	Selective agents
Antibiotics		
npt II	Neomycin phosphotransferase II	Kanamycin Neomycin Geneticin (G418)
hph, hpt	Hygromycin phosphotransferase	Hygromycin B
eecC3, aacC4	Gentamycin-3-*N*-acetyltransferase	Gentamycin
ble	not known	Bleomycin Phleomycm
dhfr	Dihydrofolate reductase	Methotrexate
SPT	Streptomycin phosphotransferase	Streptomycin
aadA	16S rRNA Aminoglycoside-3'-adenyltransferase	Spectinomycin Streptomycin
Herbicides		
ber	Phosphinothricin acetytransferase	Phosphinothricin
als	Mutant forms of acetolactate synthase	Chlorsulfuron Imidazohnones
aroA	5-Enolpyruvylshikimate-3-phosphate synthase	Glyphosate (Roundup)

Due to the limited numbers of selectable marker genes, it is difficult to introduce the second gene into a transgenic plant that already contains a selectable marker gene. This factor therefore limits the production of transgenic lines with multiple desirable genes or traits, since the presence of the already existed marker gene in the transgenic lines prevents the use of the same marker in the selection of doubled-transformed plants. In order to overcome this problem, several approaches have been developed. One is called "positive selection", by which the transformed plant cells are selected since they contain an enzyme for conversion of a compound inducing a positive response such as growth or shoot formation. By this mean, only the transformed cells would have the ability to regenerate and grow on selective medium. However, the non-transgenic cells still remain alive without further growth or shoot formation (Joersbo & Okkels, 1996; Zhang *et al.*, 2000).

A good example of positive selectable marker gene is phosphomannose isomerase gene (*pmi:manA*) from *E. coli*, which utilizes mannose as a selectable agent (Negrotto *et al.*, 2000). In plants, mannose is readily taken up by roots and phosphorylated by a hexokinase to form mannose-6-phosphate. The mannose-6-phosphate is not further utilized due to the lack of phosphomannose isomerase required for its conversion to fructose-6-phosphate. The presence of *pmi:manA* gene in transformed cells therefore allows the plant to use mannose as a carbon source and grow on mannose-containing media. Since each marker gene has distinct properties, it should be evaluated independently prior to replace antibiotic or herbicide resistance genes. Although the presence of positive selectable marker genes does not cause such a big concern on further utilization of the transgenic plants that contain them, it would be more valuable if the marker gene could be removed after transformation. Subsequently, gene pyramiding by repeated transformation could be performed using the same marker gene.

Public concerns regarding the presence of antibiotic resistance genes or even non-native genes have become an important factor affecting the release of transgenic plants for commercial proposes. As a result, lengthy risk assessments are required before the crops that contain useful novel traits could be released. Therefore, the production of marker-free transgenic plants, as reviewed by Hohn *et al.* (2001), should be lessen the public concern on safety utilization of transgenic plants. Among

all the systems developed to date, the Multi-Auto-Transformation (MAT) vector developed by Ebinuma *et al.* (2000) was claimed to have various advantages over the others because the marker-free transgenic lines can be easily obtained by continuous subculture in medium with no antibiotic (Endo *et al.*, 2002; Sugita *et al.*, 1999). This system is suitable for gene transfer into plant species, particularly cassava, in which its reproduction relies on vegetative propagation.

Additionally, visible markers e.g. GUS (Jefferson *et al.*, 1987), luciferase (Ow *et al.*, 1986) and green fluorescent protein (GFP) (Chalfie *et al.*, 1994) have been used for selection of transgenic plants. Among these, the most frequently used reporter gene in plant transformation is GUS. In some plant species, the high activity of β-galactosidase could be destructive to the tissue, thus leading to the development of a non-destructive assay, which is based on secreted GUS in spent media of *in vitro* plant culture. This system is advantageous since it can be used to detect the presence of GUS in particular cell or tissue type.

5. Starch biosynthesis

In terms of the amount made, the universality of its distribution among plant species, and its commercial importance, starch is the most significant form of carbon reserve in plants. It consists of different glucose polymers arranged into a three-dimensional, semi-crystalline structure (Figure 2.4). The biosynthesis of starch involves not only the production of the composite glucans but also the arrangement of these glucans into an organized form within the starch granule. Although the formation of starch granule can be viewed as a simple model, complete details regarding the biochemical processes of starch granule assembly are not yet understood. One emerging concept is that structure within the granule itself may determine or influence the way in which starch polymers are synthesized.

Starch is synthesized in plastids. The type of plastid that commits primarily to starch production is called amyloplast, which develops directly from proplastid and has little internal lamellar structure. Starch may also be synthesized in plastids that have other specialized functions, such as chloroplasts (photosynthetic carbon fixation), plastids of oilseed (fatty acid biosynthesis), and chromoplasts of roots such

as in carrot (carotenoid biosynthesis). In some cases, for example, in the cotyledons of some legumes, amyloplasts can also develop from chloroplasts. These amyloplasts may maintain considerable amounts of stacked lamellar material from the thylakoids and, in cells receiving sufficient light, may undertake some photosynthetic carbon fixation to use as substrate for starch biosynthesis.

Starch is composed of two glucan polymers: amylose, a predominantly linear α-1,4-linked glucan, and amylopectin, α-1,4-linked glucan chains that are joined together by α-1,6 linkages (Figure 2.4C). In higher plants, starch is made in chloroplasts of leaves (transitory starch) and in amyloplasts of non-photosynthetic storage organs (reserve or storage starch). The small, disc-shaped granules of transitory starch from various plant species show little variation. In contrast, the size and shape of storage starch granules are extremely diverse depending on plant species.

The biosynthesis of starch requires three enzymatic activities; ADPglucose pyrophosphorylase, starch synthase, and starch-branching enzyme. The ADP-glucose pyrophosphorylase or AGPase (glucose-1-phosphate adenylyltransferase; EC 2.7.7.27) catalyzes ADP-glucose synthesis. This reaction is allosterically regulated by 3-phosphoglyceric acid (3PGA) as an activator and PP_i as an inhibitor (Mukerjea *et al.*, 2002). Various mutants that have a defect in AGPase activity exhibit severe deficiencies in the rate and ability to synthesize starch. As a result, AGPase has been recognized as rate-limiting enzyme for the starch biosynthesis pathway. In plants, AGPase exists as heterotetrameric protein ($\alpha_2\beta_2$) composing of a pair of large subunits and a pair of small subunits, which share high homology but are still distinguishable by their amino acid sequences (Smith & Preiss, 1992). Overexpression of an AGPase gene in cassava could result in an enhancement the total root biomass accumulation (Ihemere *et al.*, 2006).

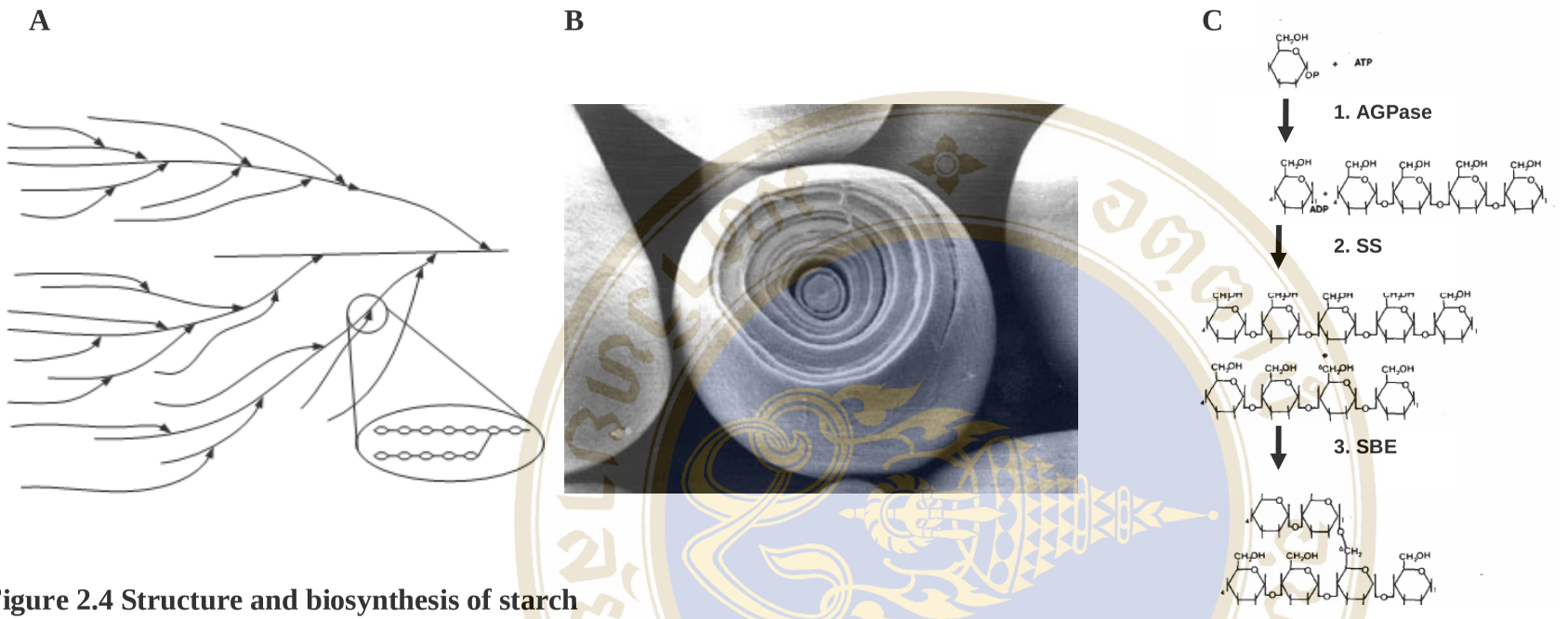


Figure 2.4 Structure and biosynthesis of starch

(A) Diagrammatic representation of an amylopectin molecule. Chains of α-1,4-linked glucans are attached by α-1,6 linkages to form a highly branched structure. (B) Cross-section of a starch granule showing alternating semicrystalline and amorphous growth rings. (C) Steps of starch biosynthesis composing of the ADP-glucose formation by AGPase (step 1), the elongation of glucan chains by SSs (step 2), and the introduction of branches by SBEs (step 3) (Martin & Smith, 1995).

Starch synthases (ADP-glucose:1,4-α-D-glucan 4-α-D-glucosyltransferase; EC 2.4.1.21) catalyze the transfer of glucose from ADP-Glc to the non-reducing end of glucan chains via an α-1,4 linkage. An isoform of starch synthase, namely granule-bound starch synthase I (GBSSI), is bound to starch granules, while the others are localized in the plastid stroma and, to some extent, bound to starch granules. GBSSI, which is also known as *waxy* protein, is a protein with average molecular mass of 60 kDa (Dry *et al.*, 1992; Sano, 1984; Shure *et al.*, 1983; Vos-Scheperkeuter *et al.*, 1986). In the *waxy* mutants of maize, a dramatic reduction in amylose content was related to the loss of GBSS activity and the absence of 58-60 kDa *waxy* protein (McDonald & Preiss, 1985). Analogous mutants have been identified in other plant species, such as glutinous rice (Sano, 1984), potato (Hovenkamp-Hermelink *et al.*, 1987), and cassava (Raemakers *et al.*, 2005). In pea, not only GBSSI but a 77-kDa protein was also observed to bind to the starch granules. Since this protein is antigenically related, though only weakly, to the 60-kDa GBSSI from potato, it has been proposed to represent a second isoform of GBSS (GBSSII) (Dry *et al.*, 1992). In cassava, two isoforms of GBSS; GBSSI (Salehuzzaman *et al.*, 1993) and GBSSII (Munyikwa *et al.*, 1997), have been identified. The GBSSI cDNA encodes a polypeptide of 608 amino acids, which includes the N-terminal chloroplastic transit peptide of approximately 8 kDa. After chloroplastic translocation, a mature protein of 59 kDa (530 amino acids) would be obtained. The cassava GBSSI cDNA shows a high degree of sequence similarity to GBSSI from various plants (60-74%). According to Southern analysis, the cassava GBSSI is a low copy number gene. The gene is expressed in a number of different organs, but most abundantly in the tubers (Salehuzzaman *et al.*, 1993).

In addition to GBSS, multi-sequence alignment analysis of the starch synthase proteins from various plant species identified four additional classes of starch synthase; SSI, SSII, SSIII and SSIV. In rice, one gene for SSI, three for SSII and two each for SSIII, SSIV and GBSS were identified (Hirose & Terao, 2004). The soluble starch synthases of pea are composed of two isoforms; a 77-kDa protein and an immunologically cross-reactive protein of 60 kDa. Both of these isoforms show similar kinetic properties (Smith & Denyer, 1992). In cassava, two isoforms of

soluble starch synthase, namely SSI and SSII, have been observed (Netrphan *et al.*, personal communication).

The last enzyme in the starch biosynthesis pathway is starch branching enzyme (Q-enzyme or 1,4-α-D-glucan:1-4-α-D-glucan-6-glycosyltransferase; EC 2.4.1.18). It catalyzes the formation of branch points in starch by breaking an α-1,4 glycosidic bond followed by transfer of the cut glucan to C_6 of another glucose residue in the same or different chain. Two major isoforms of starch branching enzymes, SBEI and SBEII, have been observed in plants. Additionally, the maize SBEII has further been classified into two subgroups; SBEIIa and SBEIIb (Gao *et al.*, 1997). Unlike GBSSI and GBSSII, the SBEI and SBEII are immunologically distinct. Based on their substrate specificities, SBEI branches amylose 20 times faster than amylopectin, while SBEII branches amylopectin twice as fast as amylose and 6 times faster than SBEI does (Guan & Preiss, 1993). This evidence therefore suggests that SBEI produces the initial branches, and these slightly branched polymers become the substrate of SBEII. In pea, a mutant lacking only SBEIIb activity has been identified (Bhattacharyya *et al.*, 1990). The amount of starch in this mutant was equivalent to 50% of that in wild type plants and the rest of unused glucose equivalents remained in the form of sucrose. Furthermore, the residual amylopectin also appeared to contain less branches and more long chains.

6. Inhibition of plant gene expression by RNA interference

Post-transcriptional gene silencing (PTGS) is a control mechanism that results in degradation of specific mRNA sequences. It includes antisense-mediated gene silencing, co-suppression, RNA interference (RNAi) and virus-induced gene silencing. The plants PTGS plays a key role to control pathogenic RNA sequences such as those from viruses and viroids. This phenomenon was initially described in transgenic plants that contained extra copies of dihydroflavonol-4-reductase (*dfr*) or chalcone synthase (*chs*) but still lost expression of the corresponding endogenous genes (Krol *et al.*, 1990). This observation was termed co-suppression since the RNA species derived from both transgene and homologous endogenous genes were degraded. Similar findings were described in *Neurospora crassa*, in which the

phenomenon was called quelling (Cogoni & Macino, 1999), and in animals, in which it was referred to as RNAi (Figure 2.5) (Fire *et al.*, 1998).

To induce gene silencing via RNAi mechanism, a vector containing inverted repeat sequences must be introduced into host cells. An important feature of these inverted repeat constructs is the insertion of a spacer, which provides stability in *E. coli* and *Agrobacterium* (Waterhouse *et al.*, 1998). A spacer of only 150 bp was found to be sufficient for stable cloning of the inverted repeat sequences. By replacing the spacer with a spliceable intron, the silencing efficiency was increased dramatically (Smith *et al,* 2000). To efficiently induce gene silencing, an intron is often included in the construct used in plant transformation. The RNAi has been used to study the function of genes in various plants, for example, by using versatile-type RNAi vectors, driven by constitutive promoters. Although these vectors are highly useful, constitutive suppression of the target gene can sometimes result in plant death or seed abortion. To solve this problem, RNAi vectors containing inducible or tissue-specific promoter have been developed for high-throughput functional genomics. By using tissue-specific promoter, spatial disruption of the target gene could be obtained. This system is useful for dissecting function of genes involved in organ formation (Byzova *et al.*, 2004). Tissue- or organ-specific RNAi is useful not only for gene function research but also for a crop improvement program, for example, to produce oil seeds with altered fatty acid composition (Stoutjesdijk *et al.*, 2002) and tomato with high carotenoid and flavonoid contents (Davuluri *et al.*, 2005).

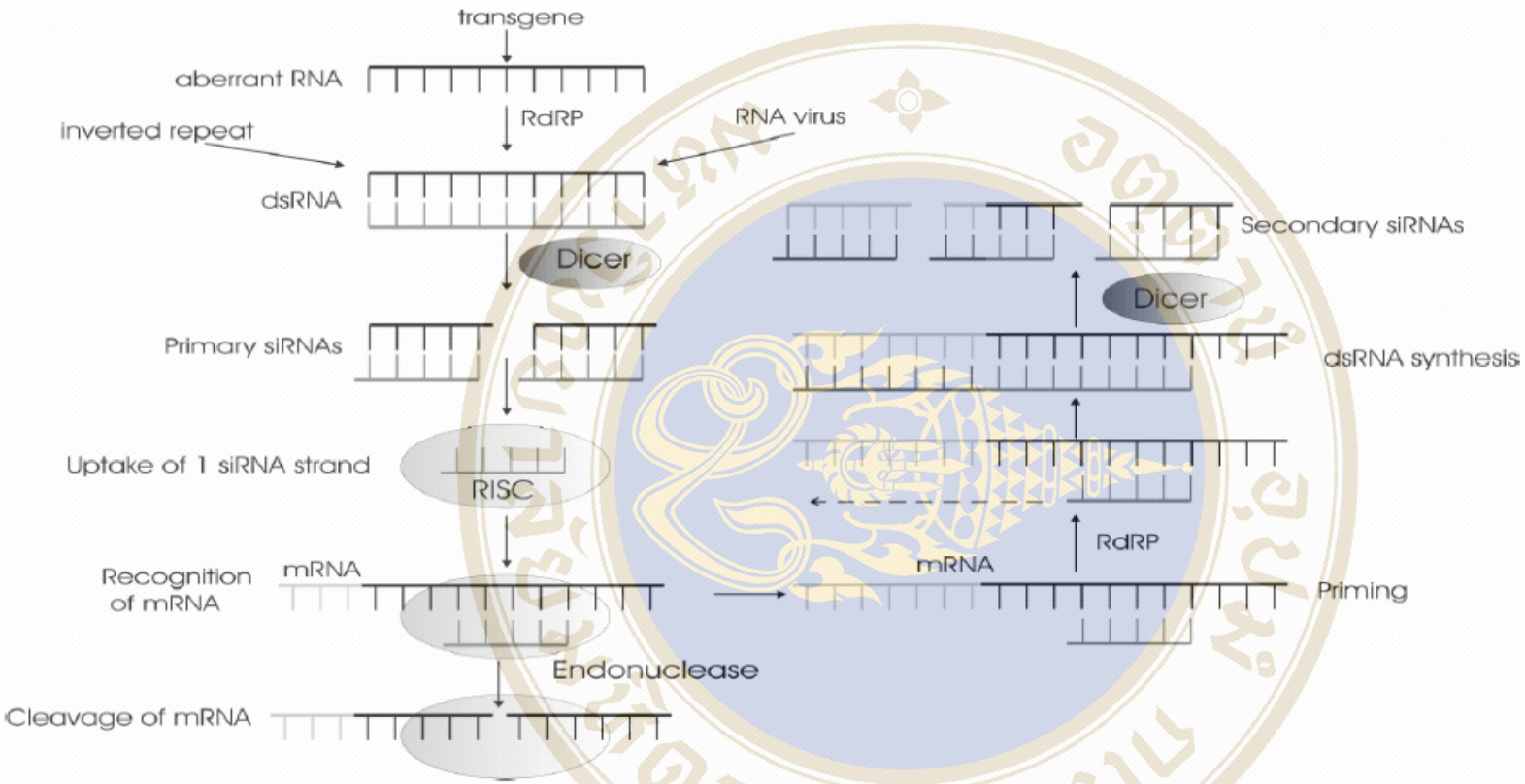


Figure 2.5 Mechanisms of RNAi-induced gene silencing

Double-stranded RNA is digested by enzyme called dicer to yield uniformly sized small interfering (si) RNA. The double-stranded siRNA is a component in RNA-induced silencing complex (RISC) catalyzing the degradation of a specific RNA sequence. Additionally, the siRNA can act as a primer to facilitate the synthesis of dsRNA via RNA-dependent RNA polymerase (RdRP)-mediated pathway to yield secondary siRNAs (Heilersig *et al.*, 2006).

7. Cassava genetic transformation

Various reports on cassava transformation are currently available (Table 2.2). This technique can be used to complement traditional breeding, thus resulting in the production of transgenic with various desirable traits, for example, pest and disease resistance, high yield and improved nutritional quality.

According to a world-wide survey, the diseases that are currently affect cassava are caused by over 30 different types of fungi and bacteria. In Latin America, the cassava common mosaic virus (CsCMV) is the main cause in a dramatic loss of cassava yield. Therefore, CsCMV-resistant cassava was produced (Schopke *et al.*, 2000). Due to its long growing cycle, 8-24 months, cassava is also subjected to repeated and prolonged attacks from various insect pests including whiteflies, mites, mealybugs and hornworms. These pests have been reported to be the cause for the loss in yield and planting material in Africa, Asia and South America (Belloti *et al.*, 1999). To obtain plants with resistance to insect pests, *cry* genes, which encode insect specific endotoxins (*Bt* toxin) has been introduced into cassava (Schnepf *et al.*, 1998) Expression of the *cry* genes in transgenic cassava would complement the methods that are currently available for pest control.

In many regions of the world, cassava is used as staple food. For safe consumption of cassava, both as food and feed, its cyanide content needs to be reduced. Traditionally, this could be done by maceration, soaking, rinsing and baking; however, short-cut processing techniques can yield toxic food products. With an advancement in genetic engineering, transgenic cassava plants with reduced linamarin content, the major form of cyanogenic glucoside in cassava, was produced (Siritunga & Sayre, 2003). It was found that the linamarin content in the roots and leaves of transgenic cassava was reduced by 99% and 94%, respectively. In addition to cyanogenic compounds, new cassava lines with improved nutritional value would be required. To increase the root protein content, lines expressing artificial storage protein ASP1 were produced (Zhang *et al.*, 2003b). Additionally, the BioCassava Plus project, led by Dr.Richard Sayre – a professor of plant cellular and molecular biology at Ohio State University, is currently being carried out with the main objective to increase not only protein but mineral and vitamin contents of cassava.

Table 2.2 Cassava transformation experiments (Zhang *et al*., 2000)

Explant	Regeneration mode	Gene transfer technique	Plasmid (genes)	Selection	Transgenic tissues	Expression
somatic embryo	SE	electroporation	pDE4(*Uid*A)	___	chimeric transformed embryos	TGE
somatic cotyledon	SO	*Agrobacterium*	pTOK233 (*hpt*, *uid*Aint, *nptII*); pBinGusInt(*nptII*, *uid*Aint)	hygromycin	transgenic plants	SAP; NAP
suspension	SE	particle bombardment	pILTAB313(*nptII*, *uid*A)	paromomycin	transgenic plants	SAP
suspension	SE	particle bombardment	pJIT100(*luc*, *pat*); pJIT64(*luc*)	luciferase	transgenic plants	SAP
suspension	SE	particle bombardment	pILTAB28(*uid*A)	___	chimeric transformed suspension cells	TGE
suspension	SE	particle bombardment	pHB1(*luc*, *AGPase*); pJIT100(*luc*, *pat*)	luciferase and ppt	transgenic plants	SAP; NAP
suspension	SE	*Agrobacterium*	pMON977(*nptII*, *uid*Aint)	paromomycin	transgenic plants	SAP
somatic cotyledon	SE	*Agrobacterium*	pGV1040(*nptII*, *bar*, *uid*A)	ppt	transgenic plants	SAP

Abbreviations: SE = somatic embryogenesis, SO = shoot organogenesis, TGE = transient gene expression, SAP = Southern analysis positive, NAP = Northern analysis positive, For gene abbreviations see text.

In this study, tissue culture and transformation systems suitable for Thai cassava cultivars would be established. Inhibition of endogenous expression of a starch biosynthetic gene by RNAi mechanism would be carried out. Since the systems developed in this study could be used as a platform to study the function of other starch biosynthetic genes in cassava, it would therefore be rational to have a tissue-specific promoter that could be used to direct expression of the foreign gene into the cassava storage roots. Unfortunately, a cassava root-specific promoter is not currently available; however, the most suitable promoters would be those of the c15 and c54 genes. The c15 gene encodes a protein that showed high degree of similarity to cytochrome P_{450} from *Sorghum bicolor* (52% identity, accession no. 048958) and *Arabidopsis* (43% identity, accession no. NP_200536). Deduced amino acid of the c54 gene should represent a glutamic acid-rich protein in cassava (accession no. AY101376). According to Zhang *et al.* (2003a), the promoter regions of c15 and c54 (namely p15 and p54, respectively) were shown to predominantly active in phloem, cambium and xylem vessels of vascular tissues from leaves, stems, and root systems in both cassava and *Arabidopsis*. In cassava, strong expression of the *uidA* reporter gene was observed in the starch rich parenchyma cells of the roots. The results therefore suggest that targeted expression of genes in storage root tissue of cassava could be conducted using the p15 and p54 promoter. Based on the results mentioned above, these promoters are also functional in *Arabidopsis*. In this study, the function of these promoters would be tested in other model plants potato and tobacco. If these promoters could behave in potato the same way they do in cassava, comparative analysis of the starch biosynthetic genes could be carried out using the same constructs made for cassava. Availability of these systems should help us to better understand starch biosynthesis pathway in tuber crops, thus leading to an efficient improvement of starch quality and quantity via genetic engineering in the future.

CHAPTER III
MATERIALS AND METHODS

1. Chemicals and reagents

Chemicals and reagents used throughout this study were molecular biology grade, analytical grade and tissue culture grade and purchased from Sigma-Aldrich (USA), Fluka (USA), Merck (Germany), *Phyto*Technology Laboratories (USA) and Roche (USA). These included absolute ethanol, acetosyringone (3,5-dimethoxy-4-hydroxy-acetophenone), agarose, bactoagar, gelrite, ammonium acetate, ammonium chloride, 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), 1-naphthaleneacetic acid (NAA), 3-indolebutyric acid (IBA), 5-bromo-4-chloro-3-indolyl-beta-D-glucoronic acid cyclohexylammonium salt (x-gluc), calcium chloride, carbenicillin, cefotaxime, hygromycin B, rifampicin, kanamycin, spectinomycin, ampicillin, augmentin, chloroform, Haiter (0.6% sodium hypochlorite solution), diethylpyrocarbonate (DEPC), dimethylsulfoxide (DMSO), disodium-ethylenediaminetetraacetic acid (EDTA), ethidium bromide, glycerol, glycine, hexadecyl trimethyl ammonium bromide (CTAB), hydrochloric acid, hydroxyquinoline, isopropanol, kinetin, phenol, magnesium chloride, magnesium sulfate, 2-mercaptoethanol, *myo*-inositol, nicotinic acid, potassium acetate, potassium chloride, potassium ferricyanide, potassium ferrocyanide, L-proline, sodium chloride, sodium dodecyl sulfate (SDS), sodium hydroxide, sodium phosphate, spermidine free base, sucrose, thiamine, tris (hydroxymethyl) aminomethane, Triton X-100 and Tween 20.

Molecular biology products were purchased from Fermentas (USA), Invitrogen (USA), New England Biolabs (USA), Promega (USA) and QIAGEN (USA). Molecular weight DNA ladders were obtained from Fermentas (USA). DNA-modifying enzymes were obtained from New England Biolabs (USA). *Taq* DNA

polymerase, 10x PCR buffer and $MgCl_2$ were obtained from Fermentas (USA). RT-PCR products were obtained from Invitrogen (USA). Hybond ^{TM}-N+ was purchased from GE Healthcare (Sweden). X-ray films were obtained from Kodak (Australia).

2. Plant materials

2.1 Tobacco plants

Axenic culture of the *Nicotiana tabacum* L. cultivar Ottawa was prepared from seeds. After the seeds were sterilized in 10% v/v Haiter (0.6% sodium hypochlorite solution) for 15-20 min and washed four times with sterile water, they were germinated on RM medium (Murashige and Skoog (MS) medium with 3% sucrose, solidified with 6 g/l bacto agar) at 26^{o}C under a 16-hr photoperiod of 3,000 lux for 3-4 weeks. The cultures were maintained by subculture of the node cuttings at 4 weeks interval.

2.2 Potato plants

Axenic culture of the *Solanum tuberosum* cultivar Patrone was kindly provided by Dr. Salak Phansiri (Scientific Equipment Center, KURDI, Kasetsart University). The node-cuttings were cultured on potato basic medium (PBM) (MS medium with 2 % sucrose, solidified with 6 g/l bacto agar) at 26^{o}C under a 16-hr photoperiod of 3,000 lux for 4 weeks.

2.3 Cassava plants

Stems with shoot apexs and lateral buds of cassava (*Manihot esculenta* Crantz) cultivar Kasetsart 50 (KU 50) and Hanatee were harvested from Rayong Field Crops Research Center, surface sterilized for 15-20 min in 10% v/v Haiter and cultured on cassava basic medium (MS with 2% sucrose, 2 μM $CuSO_4$, solidified with 0.6% bacto agar (CBM, Table 3.1). The proliferated shoots were sub-cultured at 60 days interval onto CBM using node cutting and kept at 26^{o}C under 16-hr photoperiod of 3,000 lux.

Table 3.1 Cassava tissue culture media

Components	CBM	CIM	CMM	COM	CEM	CTM
MS (g/l)	4.4	4.4	4.4	4.4	4.4	4.4
$CuSO_4.5H_2O$ (µM)	2	2	2	2	2	2
Sucrose (g/l)	20	20	20	20	20	100
BAP (mg/l)	-	-	0.1	1	0.4	-
2,4-D (mg/l)	-	12	-	-	-	-
IBA (mg/l)	-	-	-	0.5	-	-
MS organic components (1000X) (ml/l)	1	1	1	1	1	1
Bacto agar (g/l)	6	6	6	6	6	8

CBM : cassava basic medium

CIM : cassava somatic embryo induction medium

CMM : cassava maturation medium (from somatic embryo to form cotyledon)

COM : cassava organogenesis medium (from cotyledon to form shoot)

CEM : cassava elongation medium

CTM : cassava transformation medium

MS : Murashige and Skoog medium

BAP : Benzylaminopurine (cytokinin)

2,4-D : 2,4-Dichlorophenoxyacetic acid (auxin)

IBA : 3-Indolebutyric acid (auxin)

MS organic components : see Appendix B

3. Cassava tissue culture system

Protocols for tissue culture appropriate for Thai cassava cultivars, established by Dr. Salak Phansiri (Scientific Equipment Center, KURDI, Kasetsart University), were adopted and used throughout this study (Appendix A). In this study, somatic cotyledons of KU50 cultivar were obtained from cyclic somatic embryogenesis and used as explants in genetic transformation experiments. Details of cyclic somatic embryogenesis induction and cassava plant regeneration were followed as Saelim *et al*. (2006).

3.1 Somatic embryo induction

Cutting nodes were cultured on CBM for 1-3 months before somatic embryos could be induced. Apical buds, lateral buds and young leaf lobes were isolated and cultured in dim light at 26^{o}C on induction medium (CIM). In order to maintain vigorous growth of somatic embryos with minimal callus development, the culture was transferred to fresh CIM every second week. After two to three cycles, the somatic embryos were harvested and transferred to cassava maturation medium (CMM). The cotyledons obtained from the primary somatic embryos were cultured in dim light at 26°C on CIM to obtain cyclic somatic embryos used in gene transfer study.

3.2 Organogenesis induction

After being cultured for 4-6 weeks, the somatic embryos were harvested and transferred to CMM and cultured for 3-4 weeks. Young green cotyledons obtained from the somatic embryos were harvested, cut into 0.5-cm^2 pieces, transferred to shoot organogenesis induction medium (COM). To induce shoots or plantlets via organogenesis, the culture was kept in dim light at 26^{o}C.

3.3 Shoot organogenesis and cassava plant regeneration

Subsequently, shoot primordial was cultured in shoot elongation medium (CEM). To investigate the shoot organogenesis induction efficiency, the number of elongated shoots observed at the 4th week was recoded. To induce rooting and further growth, the elongated shoots were transferred to CBM. The plantlets obtained at this step were repeatedly transferred to CBM using node cutting at 2 months interval and kept at 26^{o}C, under a 16/8 hr photoperiod of 3,000 lux.

3.4 Induction of *in vitro* storage roots

To induce the *in vitro* root formation, uninodal segments (approximately 5–8 mm long) dissected from *in vitro* cassava plants were cultured on cassava storage root medium (MS with 5% sucrose, 3 g/l gelrite, 0.54 µM NAA, and 0.44 µM BAP; pH 5.8) at 26^{o}C under a 16/8 hr photoperiod rhythm of 3,000 lux (Medina *et al*., 2007).

3.5 Somatic callus suspension culture

Somatic callus of KU50, obtained from Dr. Budsaraporn Ngarmpanya (Silapakorn University), was cultured in MS supplemented with 3% sucrose, 1 mg/l 2,4-D, 1 mg/l BAP and 100 mg/l *myo*-inositol at pH 5.8. To allow the young callus to further develop to mature stage, the culture was kept for 2 months at 26°C under a 16/8 hr photoperiod with shaking at 110 rpm.

4. Bacterial culture and stock culture maintenance

The *E. coli* strain DH5α and TOP10 (Invitrogen) (Table 3.2) were grown at 37^{o}C for 16 hr in liquid or solid LB medium supplemented with appropriate antibiotics. Depending on the type of plasmids, kanamycin and spectinomycin at 50 mg/l and ampicillin at 100 mg/l were added to the bacterial culture medium. For *Agrobacterium tumefaciens*, three strains, AGL1, EHA105 and LBA4404, were used in this study (Table 3.2). All of them were grown at 28°C for 2-3 days in YEP

medium (Appendix B) containing 25 mg/l of rifampicin and 50 mg/l of kanamycin or spectinomycin. These bacterial cultures were kept at 4°C for 1-4 weeks for routine work.

For long-term storage, the bacteria were grown in LB at 37°C for 16 hr (for *E. coli*) or in YEP medium at 28°C for 2-3 days (for *Agrobacterium*) with shaking at 200-250 rpm. A 500-µl aliquot of the cell culture was mixed with sterile glycerol to obtain a final concentration of 20% (v/v). After frozen in liquid nitrogen, the culture was kept at -80°C.

Table 3.2 Bacterial genotypes

Bacterial strain	Genotype
E. coli DH5α	*deo*R, *end*A1, *gyr*A96, *hsd*R17 (rk^-mk^+), *rec*A1, *rel*A1, *sup*E44, *thi*-1,F^-
E. coli TOP10 (Invitrogen)	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
A. tumefaciens AGL1	AGL0 (C58 pTiBo542) *rec*A::bla, T-region deleted Mop(+) Cb(R)
A. tumefaciens EHA105	C58 pTiBo542, T-region::*aph*, Km (S) (EHA105 is a Km (S) derivative of EHA101)
A. tumefaciens LBA4404	(Ach5 pTiAch5) Sm/Sp(R) in the virulence plasmid (from Tn904); all T-DNA of pTiAch5 eliminated in pAL4404

5. Plasmids used in this study

5.1 List of plasmid vectors

5.1.1 pCAMBIA1301

The vector pCAMBIA1301 was purchased from CAMBIA Company, Australia. The T-DNA region of pCAMBIA1301 contains β-glucuronidase gene (*uidA*, *gus*), with inserted catalase intron, as a marker gene and hygromycin

resistance gene *(hyg*^{r}*, hpt)* as a selectable gene, both of which are driven by CaMV 35S promoter (Figure 3.1).

5.1.2 pCAMBIA1301::p54::*gus*

This plasmid vector was provided by Dr. Peng Zhang (Institute of Plant Sciences, Switzerland). It was constructed by replacing the CaMV 35S promoter of pCAMBIA1301 with the vascular tissue-specific promoter p54 from cassava (Zhang *et al.*, 2003a) (Figure 3.2).

5.1.3 pCAMBIA1301::p54::GBSSi::*gus*

The pCAMBIA1301::p54::GBSSi::*gus* carried inverted repeat fragments of a cassava GBSSI gene, driven by the cassava p54 promoter (Figure 3.3). Detailed construction of this vector was described in Topic 5.2.

5.1.4 pKANNIBAL::35S::GBSSi

The pKANNIBAL::35S::GBSSi (Figure 3.5) was constructed by Dr. Supatcharee Netrphan (BIOTEC) by insertion of partial GBSSI cDNA from cassava, in both forward and reverse orientations, into the two multiple cloning sites of pKANNIBAL (Figure 3.4).

5.1.5 pART27::35S::GBSSi

This plasmid vector, kindly provided by Dr. Supatcharee Netrphan (BIOTEC), was obtained by insertion of *Not*I fragment from pKANNIBAL::35S::GBSSi into *Not*I site of pART27 (Figure 3.6). As a result, the region between the left and right borders of pART27::35S::GBSSi would contain CaMV 35S promoter, GBSSI inverted repeats flanking an intron from pyruvate orthophosphate dikinase gene, and an OCS terminator sequence (Figure 3.7).

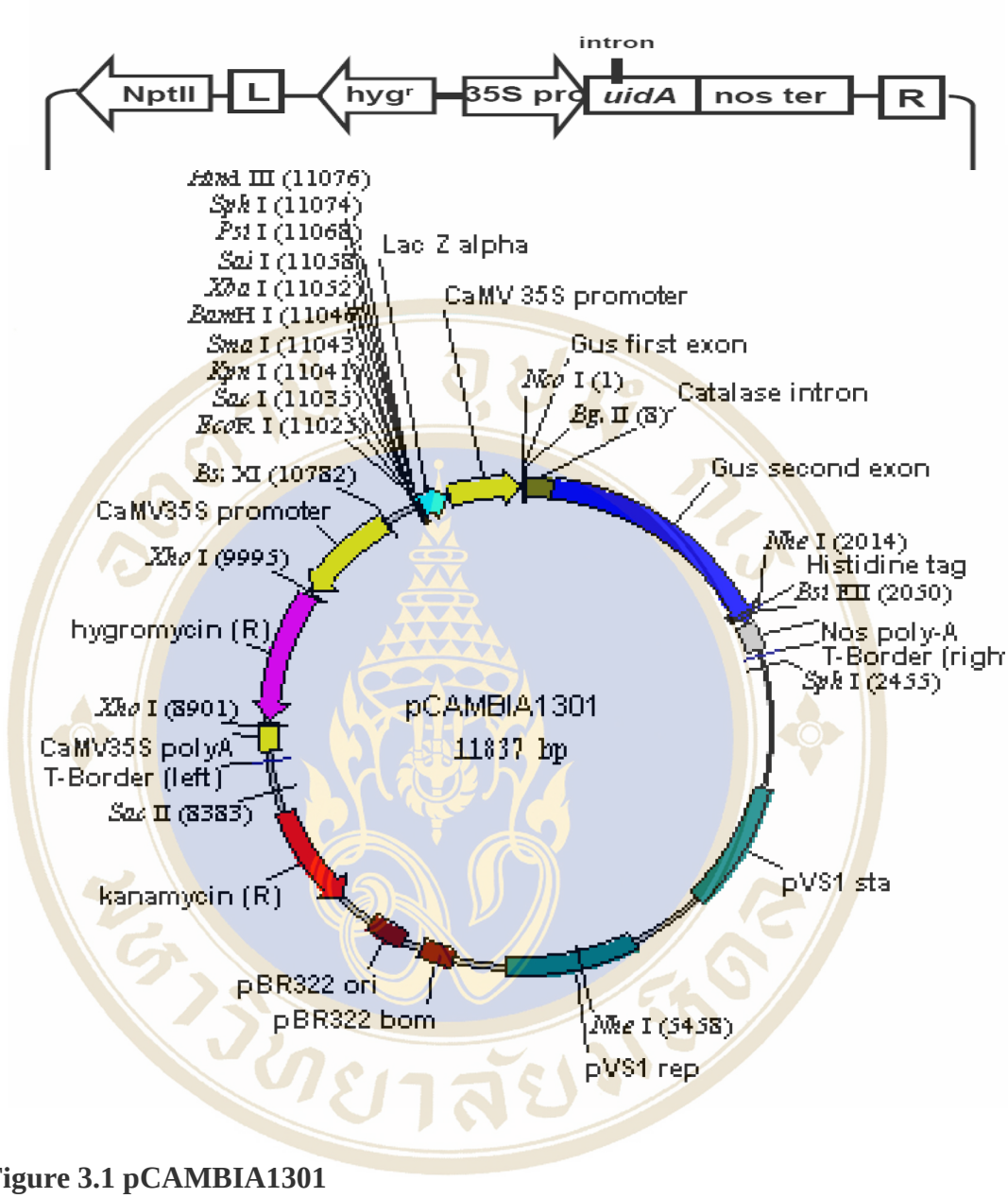


Figure 3.1 pCAMBIA1301

The T-DNA region of pCAMBIA1301 contains β-glucuronidase (*gus*) and hygromycin resistance genes, each of which is driven by separate CaMV 35S promoter.

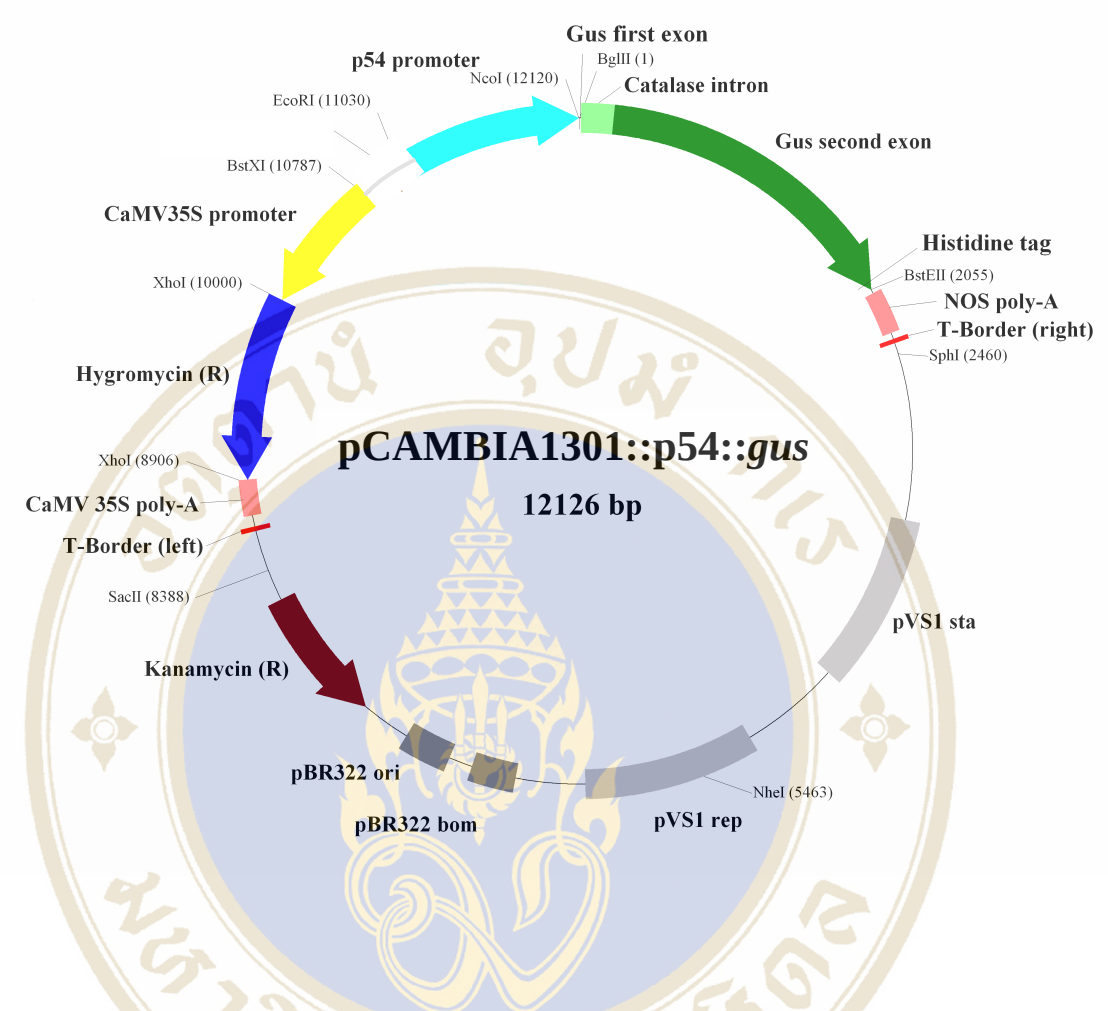


Figure 3.2 pCAMBIA1301::p54::*gus*

Compared with pCAMBIA1301, expression of GUS in the vector pCAMBIA1301::p54::GBSSi::*gus* is driven by p54 promoter.

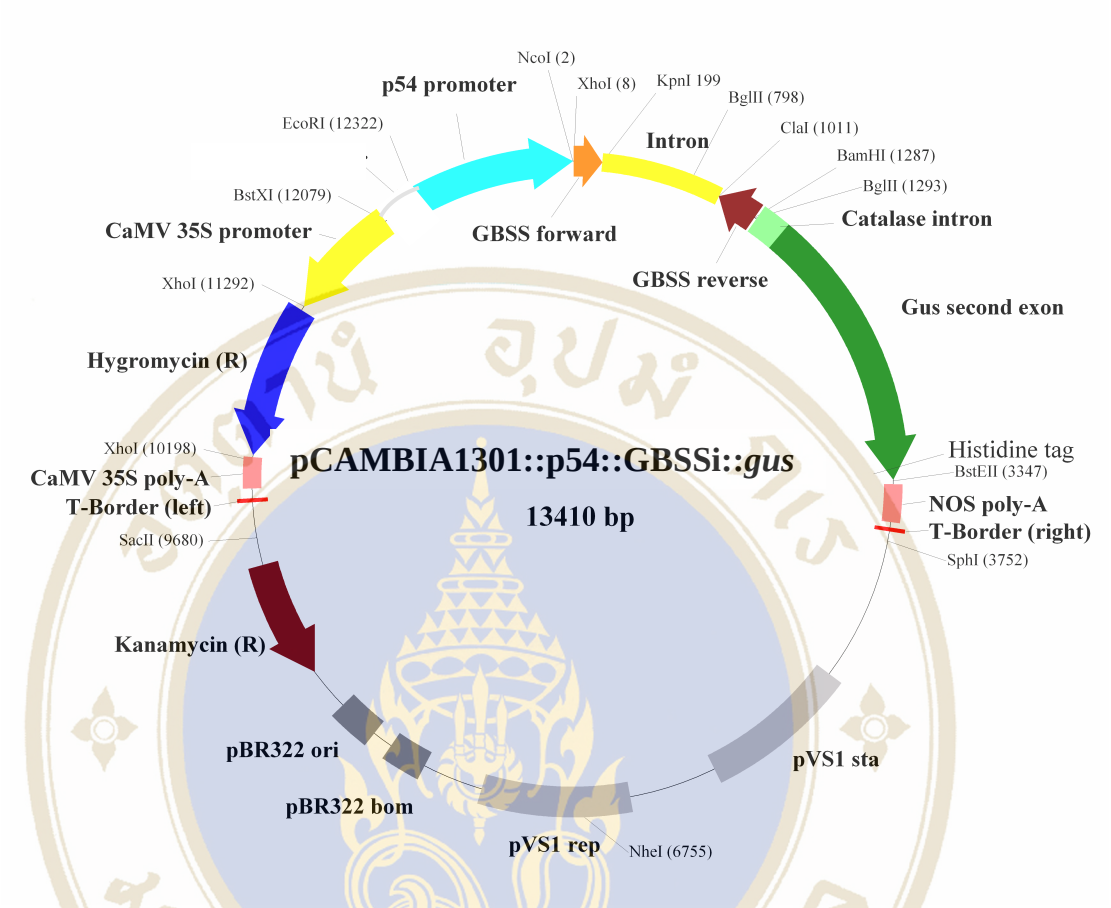


Figure 3.3 pCAMBIA1301::p54::GBSSi::*gus*

Inverted repeat fragments of a cassava GBSSI gene were cloned into pCAMBIA1301::p54::*gus* (Figure 3.2) via *Nco*I and *Bgl*II sites. Construction details of this vector were described in Topic 5.2.

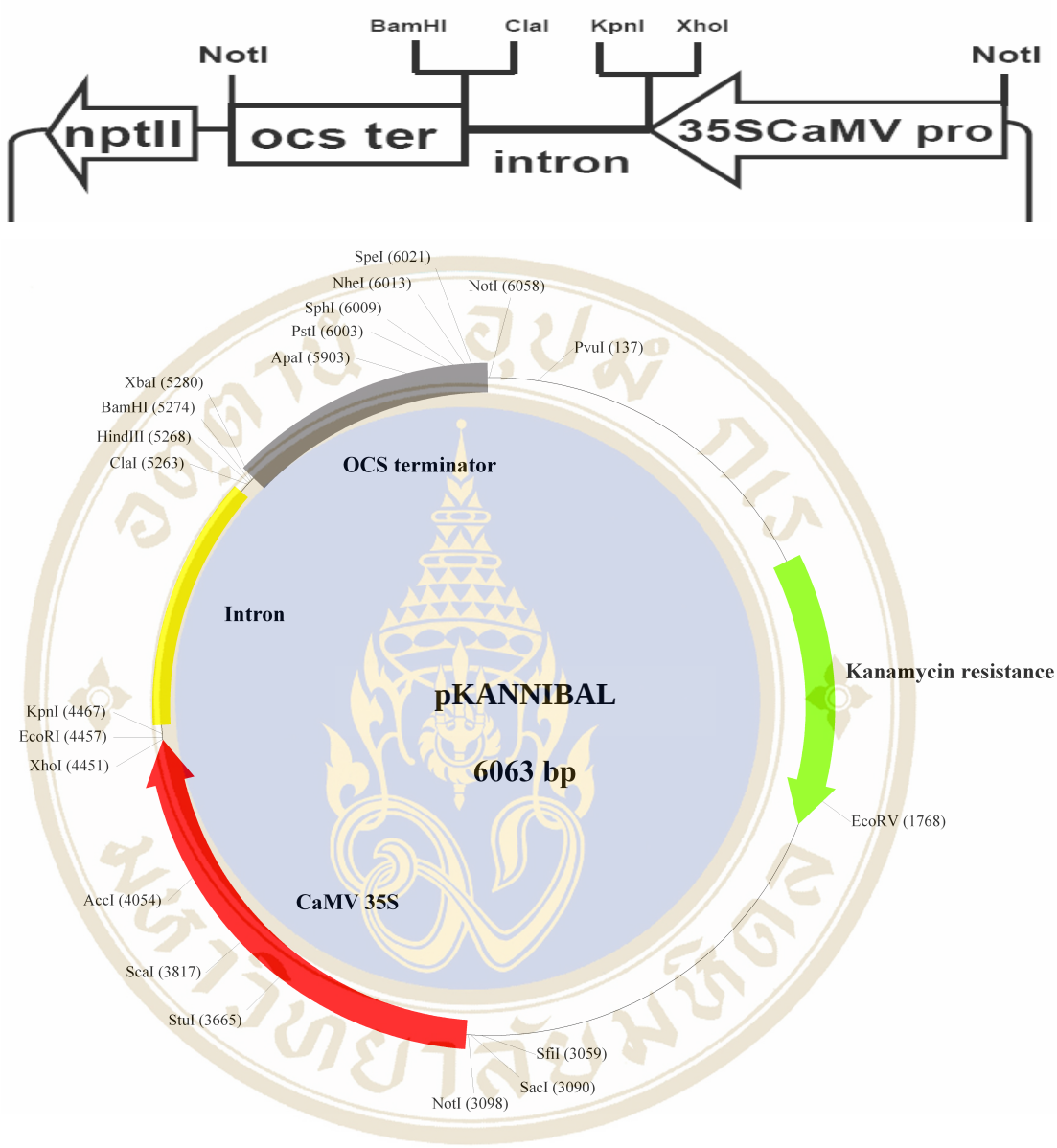


Figure 3.4 pKANNIBAL

The pKANNIBAL vector was obtained from the CSIRO Division of Plant Industry, Australia (Wesley *et al.*, 2001). It contains two multiple cloning sites suitable for cloning of inverted repeat fragments of a gene of interest.

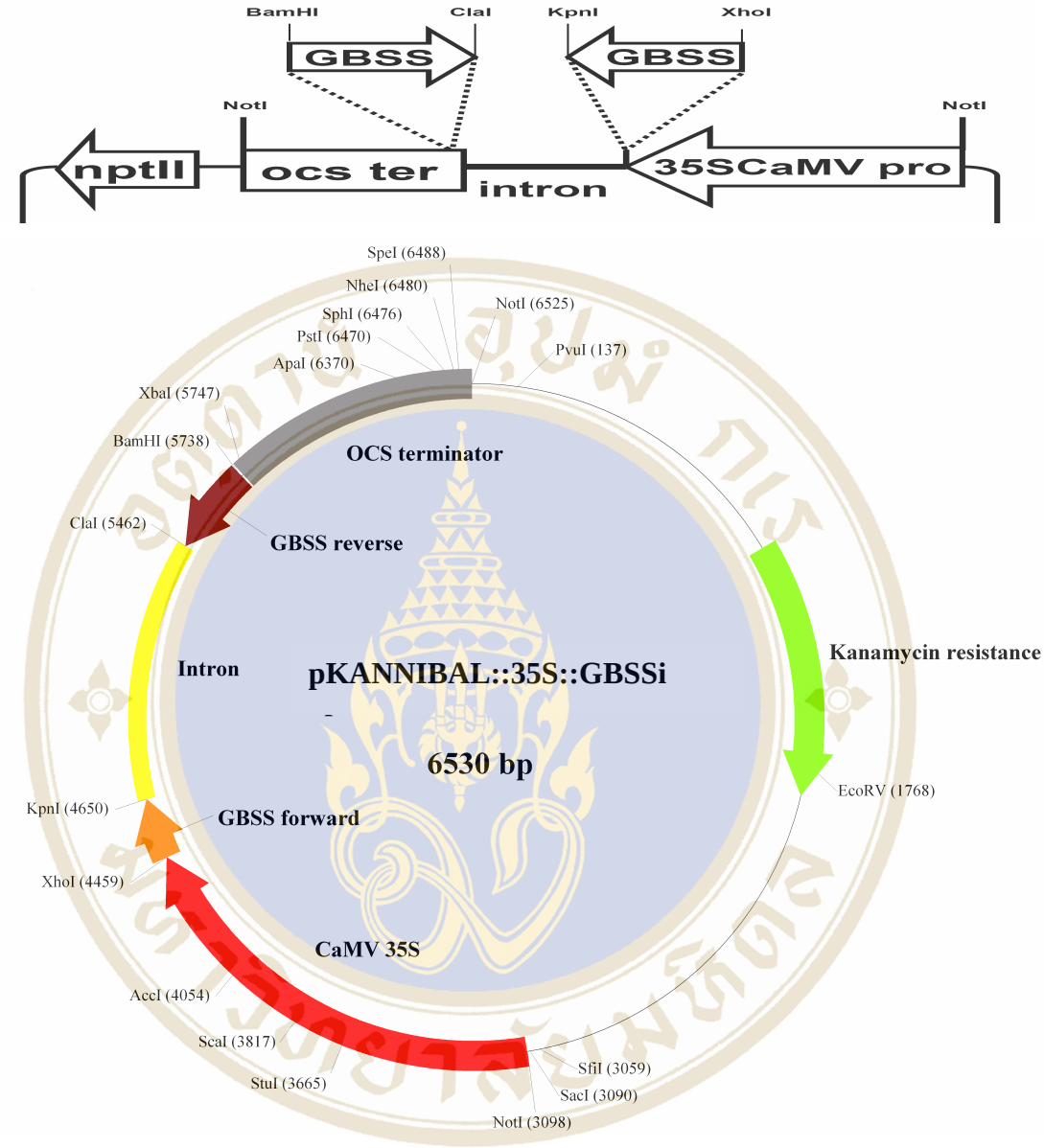


Figure 3.5 pKANNIBAL::35S::GBSSi

The pKANNIBAL::35S::GBSSi contains CaMV 35S promoter, inverted repeats of GBSSI gene fragment flanking an intron from pyruvate orthophosphate dikinase gene and octopine synthase (*ocs*) terminator.

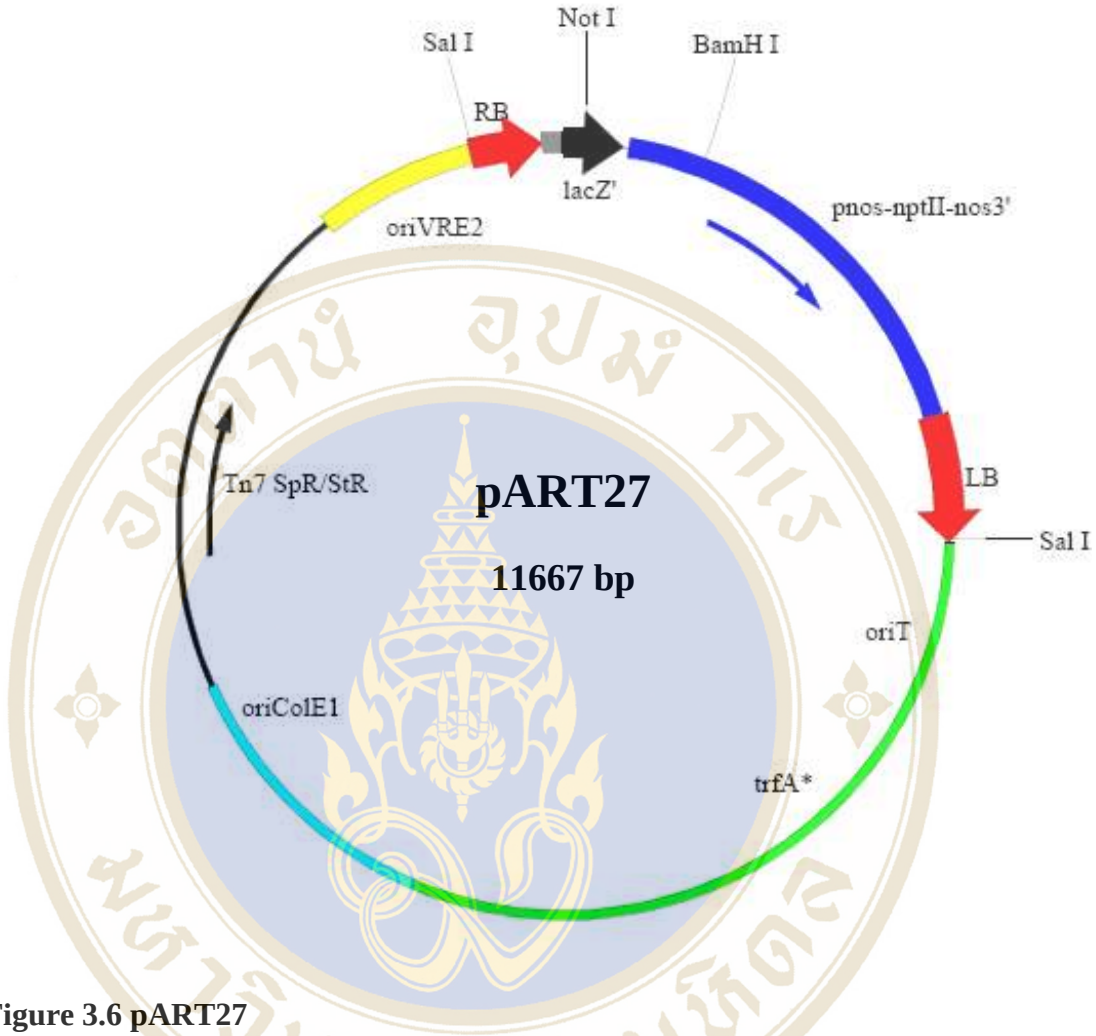


Figure 3.6 pART27

The pART27 vector was obtained from the CSIRO Division of Plant Industry, Australia (Gleave, 1992). It utilizes neomycin phosphotransferase (*nptII*) and spectinomycin resistance genes as selectable markers in plants and bacteria, respectively.

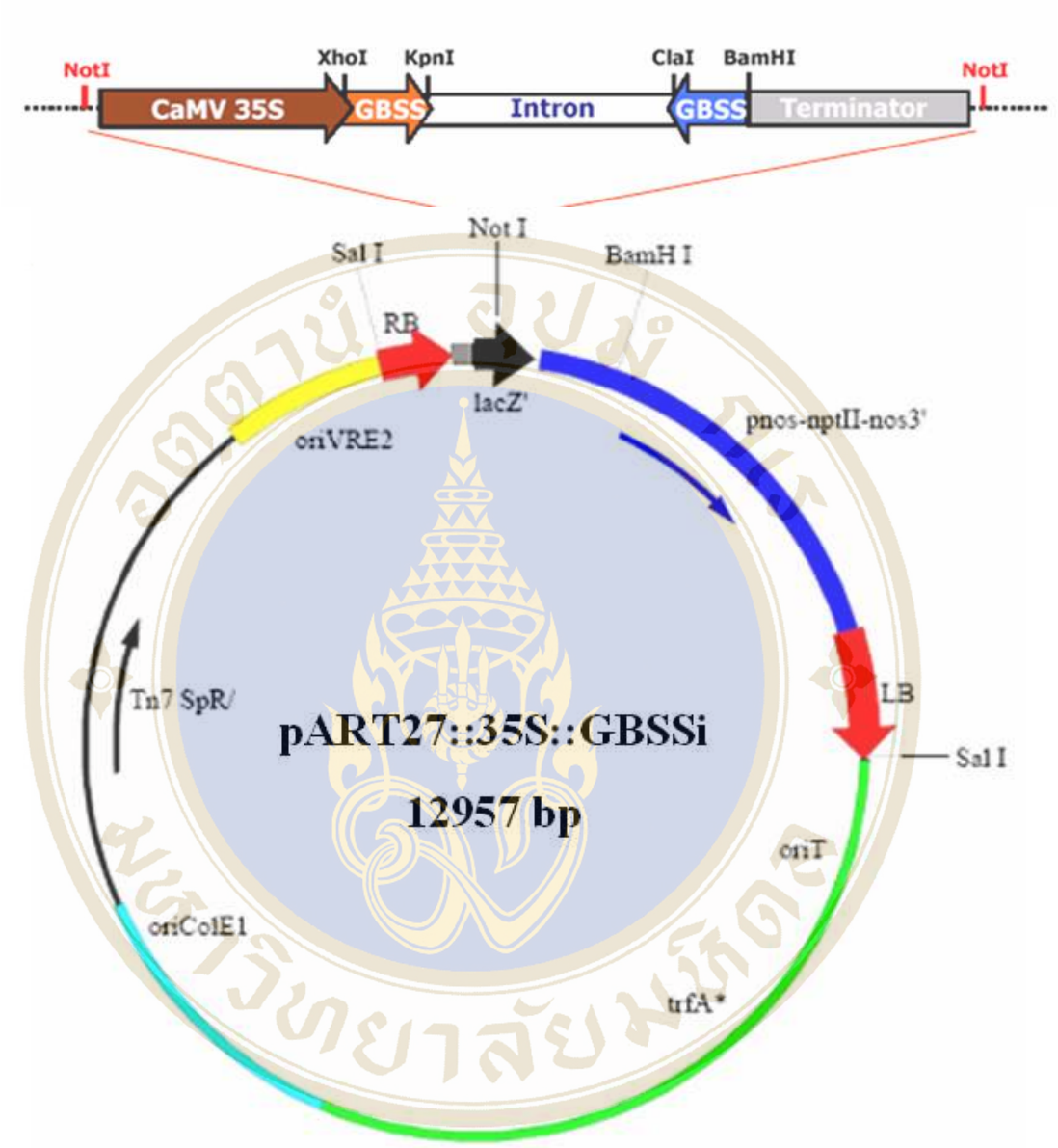


Figure 3.7 pART27::35S::GBSSi

The pART27::35S::GBSSi was constructed by ligating *Not*I-digested fragment of pKANNIBAL::35S::GBSSi into the *Not*I site of pART27. The obtained vector thus contained CaMV 35S promoter, inverted repeats of GBSSI gene fragment franking an intron from pyruvate orthophosphate dikinase gene and octopine synthase (*ocs*) terminator located within the left and right border region of pART27 vector.

5.2 Construction of the vector pCAMBIA1301::p54::GBSSi::*gus*

A fragment composing of GBSSI inverted repeats flanking an intron from pyruvate orthophosphate dikinase gene was excised from pKANNIBAL::35S::GBSSi using *Xho*I and *Bam*HI. The resulting fragment needed to be inserted into the region located between the p54 promoter and the *gus* gene of pCAMBIA1301::p54::*gus* only via *Nco*I and *Bgl*II sites. Therefore, adapters containing *Nco*I and *Bgl*II sites must be added to the 5′-*Xho*I and 3′-*Bam*HI protruding ends of the GBSSI inverted repeat fragment, respectively. The obtained fragment was digested with *Nco*I and *Bgl*II and directionally cloned into pCAMBIA1301::p54::*gus* to produce pCAMBIA1301::p54::GBSSi::*gus* (Figure 3.3).

5.2.1 Digestion of GBSSI inverted repeats from pKANNIBAL::35S::GBSSi

The pKANNIBAL::35S::GBSSi (Figure 3.5) was digested with *Xho*I and *Bam*HI to obtain a fragment composing of GBSSI inverted repeats flanking an intron from pyruvate orthophosphate dikinase gene. The fragment of 1.3 kb was subsequently cloned into the binary vector pCAMBIA1301::p54::*gus* (Figure 3.2).

5.2.2 Addition of adapters to the GBSSI inverted repeat fragment

To introduce the *Nco*I and *Bgl*II sites to the ends of GBSSI inverted repeat obtained from previous step, the oligonucleotide *Nco*I/*Xho*I-T (5′-CTATTTCCATGGC-3′), *Nco*I/*Xho*I-B (5′-TCGAGCCATGGAAATAG-3′), *Bam*HI/*Bgl*II-T (5′-GATCCAGATCTTCTA-3′) and *Bam*HI/*Bgl*II-B (5′-TAGAAGATCTG-3′) were synthesized (Figure 3.9). The *Nco*I/*Xho*I-T and *Nco*I/*Xho*I-B were annealed to produce *Nco*I/*Xho*I linker, while the *Bam*HI/*Bgl*II-T and *Bam*HI/*Bgl*II-B were used to form *Bam*HI/*Bgl*II linker. Annealing reactions were carried out by incubating the corresponding oligonucleotides at 65°C for 10 min, at 37°C for 10 min and, finally, at 25°C for 10 min. The *Nco*I/*Xho*I linker was introduced to *Xho*I site located at the 5′ end of GBSSI inverted repeat, while the

BamHI/*Bgl*II linker was introduced to *Bam*HI site located at the 3′ end of GBSSI inverted repeat. Ligation of adapters to GBSSI inverted repeat fragment was performed using 0.2 μM each of *Nco*I/*Xho*I linker and *Bam*HI/*Bgl*II linker, 80 units of T_4 DNA ligase in 1X T_4 DNA ligase buffer containing 0.5 mM ATP. The ligation was performed at 16°C for 16 hr. This would therefore yield a blunt end fragment, which could be cloned into pCR®-Blunt vector (Invitrogen) (Figure 3.10).

5.2.3 Insertion of GBSSI inverted repeats into pCAMBIA1301::p54::*gus*

For easy handling, firstly, the GBSSI inverted repeat fragment, ligated with *Nco*I/*Xho*I and BamHI/*Bgl*II linker, was cloned into pCR®-Blunt vector (Invitrogen) (Figure 3.10) and transformed into OneShot®TOP10 competent cells (Invitrogen). An intact fragment of GBSSI inverted repeats was excised from pCR®-Blunt vector by subjecting to partial digestion with *Nco*I and *Bgl*II and ligated to pCAMBIA1301::p54::*gus* that was previously digested with *Nco*I and *Bgl*II. The ligated products were transformed into *E. coli* DH5α.

Table 3.3 List of primers specific to pKANNIBAL::35S::GBSSi vector

Primer name	Sequence (5′ → 3′ direction)	Length (bp)	T_m (°C)	Expected size (bp)
GBSSi-F1	CGC GGA TCC TTT GAG CCC TGT GGTC	25	68	275
GBSSi-R1	CCA TCG ATC TGG CTG GTC CCT TCC	24	65	
GBSSi-F2	CTA TGC GAT CTC GAG CAG TTC CCA	24	61	211
GBSSi-R2	ATG CAA TTC GGT ACC ATT TCT CTT	24	55	
pKAN-F1 (A)	GTT CAT TTC ATT TGG AGA GG	20	46	267
pKAN-R1 (B)	TCC TTA CCA ATT GGG GTA C	19		
pKAN-F2 (C)	AGT TGG GAA ATT GGG TTC G	19	51	341
pKAN-R2 (D)	GCA TAT CTC ATT AAA GCA GGA CTC	24		

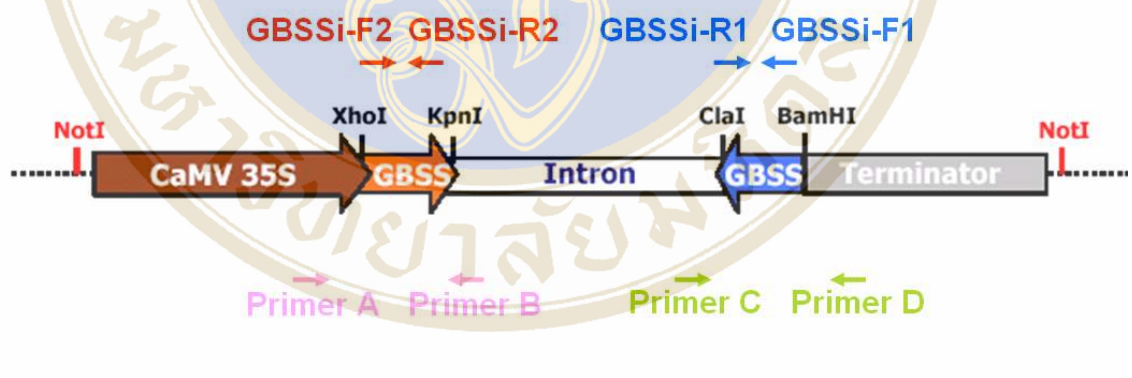
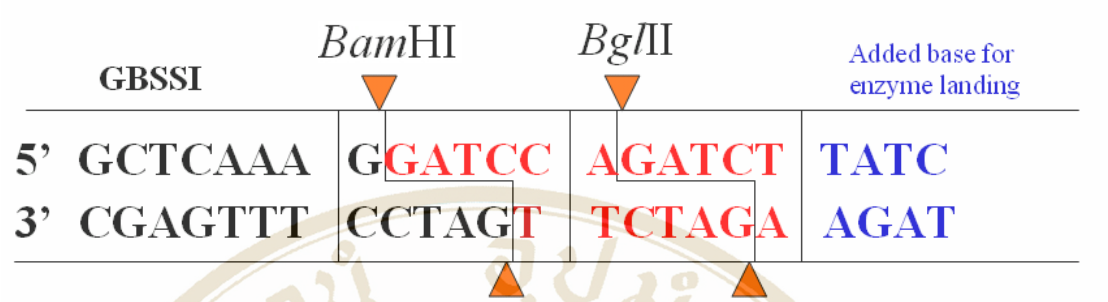


Figure 3.8 Priming sites of oligonucleotide primers on the vector pKANNIBAL::35S::GBSSi

The GBSSi primers annealed to the DNA template within GBSSI gene region, while the A, B, C and D primers bound the region outside GBSSI gene.

A. *Bam*HI/*Bgl*II adapter



B. *Nco*I/*Xho*I adapter

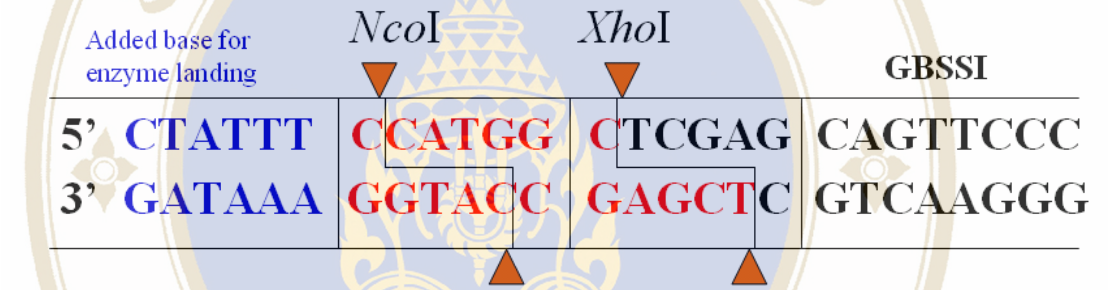


Figure 3.9 Nucleotide sequences and restriction sites of *Bam*HI/*Bgl*II and *Nco*I/*Xho*I adapters

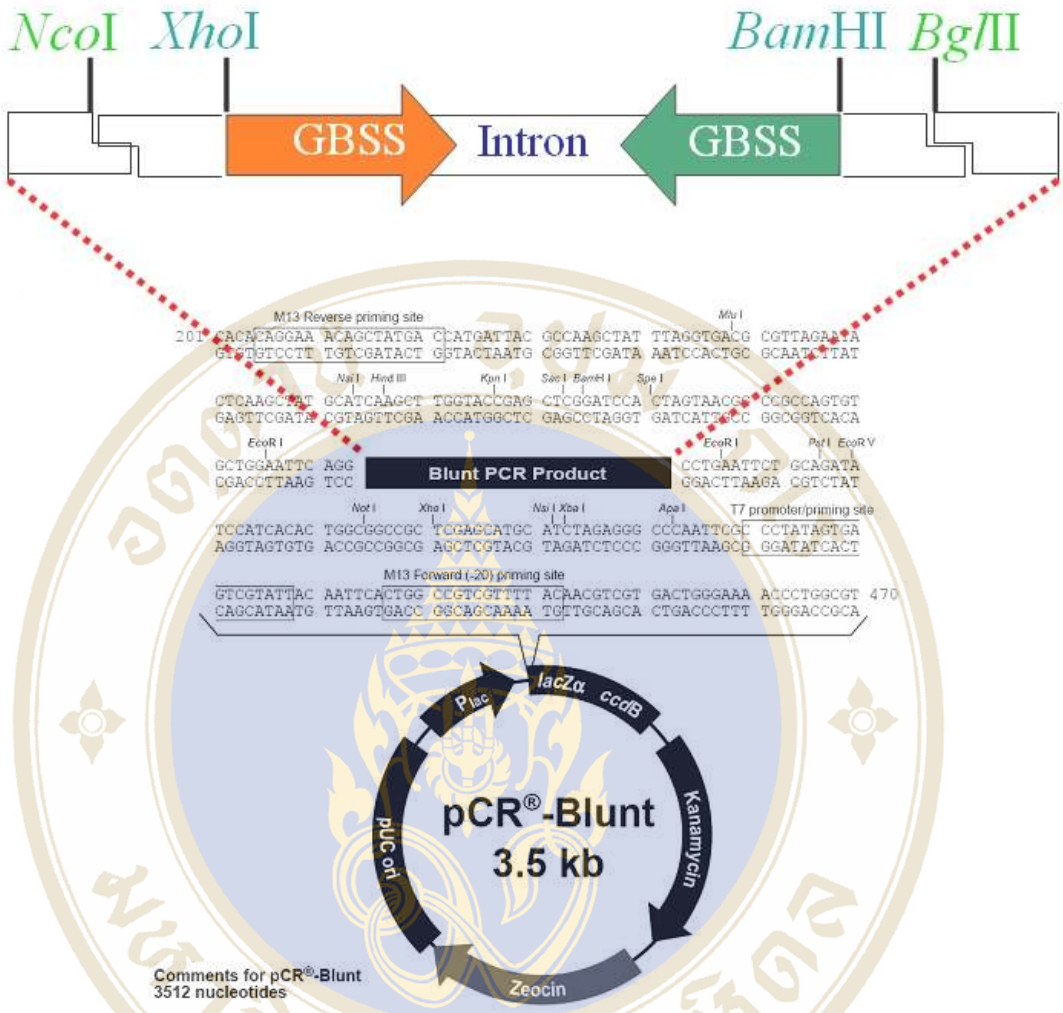


Figure 3.10 Cloning of GBSSI inverted repeat fragment into pCR®-Blunt vector (Invitrogen)

6. Plasmid DNA technology

6.1 Plasmid DNA extraction

6.1.1 Alkaline lysis method

Plasmid DNA was isolated using the rapid alkaline lysis method (Sambrook *et al*., 1989). The bacterial cell culture was centrifuged at 12,000 x g for 1 min at room temperature. The cell pellet was re-suspended in 100 µl of ice-cold solution I (50 mM glucose, 25 mM Tris-HCl; pH 8.0, 10 mM EDTA; pH 8.0) and mixed by vortexing. After the addition of 200 µl of freshly prepared solution II (0.2 N NaOH, 1% SDS), the sample was mixed by inverting a few times and then stored on ice for 5 min. This step allows immediate breakage of the bacterial cells under alkaline condition. Then, 150 µl of ice-cold solution III (3M potassium acetate, 5 M glacial acetic acid) was added and the sample was mixed by inverting the tube for 10 sec and stored on ice for 3-5 min. The cell debris was removed by centrifugation at 12,000 x g for 5 min at 4°C and the supernatant was transferred to a new tube. Plasmid DNA in the supernatant was extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The sample was vigorously mixed by vortexing, followed by centrifugation at 12,000 x g for 5 min at room temperature. The DNA in the aqueous phase was precipitated in the presence of 2 volumes of absolute ethanol and incubated for 2 min at room temperature. The DNA was collected by centrifugation at 12,000 x g for 5 min at 4°C, then washed once with 70 % ethanol and air-dried. The DNA was re-suspended 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 at 65°C for 10 min. The plasmids were then re-precipitated with absolute ethanol, re-suspended in 30 µl of TE buffer or sterile deionized water, and stored at -20°C.

6.1.2 QIAprep Spin Miniprep Kit (QIAGEN)

Bacterial cells were re-suspended in 250 µl buffer P1. After the addition of 250 µl of buffer P2 and 350 µl of buffer N3, the sample was centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was applied to QIAprep spin column followed by centrifugation at 12,000 x g for 1 min. The column was washed with 750 µl buffer PE and centrifuged at 12,000 x g for 1 min followed with additional centrifugation for 1 min to remove the residual wash buffer. The DNA was eluted by applying 50 µl of buffer EB (10 mM Tris-Cl; pH 8.5) to the column followed with centrifugation at 12,000 x g for 1 min.

6.2 Plasmid digestion with restriction endonucleases

Digestion of plasmid DNA with restriction endonucleases was performed using the conditions recommended by 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, digestion with enzyme that requires lower salt condition was performed followed with digestion with the other enzyme.

6.3 Agarose gel electrophoresis

Electrophoresis in agarose gel was performed to separate, visualize and purify DNA fragments. The concentration of agarose gel used in this study was in a range of 0.8-2.0%. To prepare the gel, agarose was mixed with 1X TAE buffer (0.04 M Tris acetate, 0.002 M EDTA; pH 8.0) and dissolved by heating. The gel mixture was allowed to cool and poured into a tray set on a gel caster of a horizontal type electrophoresis chamber (GelMate 2000). Before loaded into a well in a submarine condition, the DNA sample was mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol). Two standard markers, 100-bp and 1-kb DNA ladder (Fermentas) were fractionated along side the samples for size determination of the DNA fragments. Electrophoresis was performed in 1X TAE

buffer using a constant voltage of 100 V. The agarose gel was stained for 10-15 min with 0.5 μg/ml ethidium bromide solution. The DNA fragments were visualized on a UV transilluminator and photographed using a gel documentation system (GENE GENIOUS Bio Imaging equipped with GeneSnap software, SYNGENE).

6.4 Recovery of DNA fragments from agarose gel

A DNA fragment was purified from agarose gel using QIAquick Gel Extraction Kit (QIAGEN). A piece of agarose gel containing the desired DNA fragment was incubated in buffer QG (300 μl buffer/100 μg gel slice) at 50°C for 10 min or until the gel was completely dissolved. After the addition of isopropanol at equal volume as the gel piece, the sample mixture was applied to QIAquick spin column followed by centrifugation at 13,000 x g for 1 min. The column was washed with 750 μl of buffer PE, and centrifuged at 13,000 x g for 1 min. To elute the DNA, 50 μl of buffer EB (10 mM Tris-Cl; pH 8.5) was added to the center of the QIAquick column followed by centrifugation at 13,000 x g for 1 min.

6.5 Introduction of plasmid DNA into bacterial hosts

6.5.1 Introduction of plasmid DNA into *E. coli*

Plasmid DNA was transformed into *E. coli* using heat shock transformation method. To obtain high transformation frequency, the *E. coli* competent cells were freshly prepared using a standard $CaCl_2$ method (Sambrook et al., 1989). A 50-μl aliquot of *E. coli* competent cells was mixed with the plasmid DNA by stirring gently with a pipette tip. The mixture was incubated on ice for 20 min, placed in a 42°C waterbath for exactly 90 sec and quickly placed on ice for 2 min. After the addition of 250 μl of LB broth, the sample was incubated at 37°C for 1 hour with shaking at 250 rpm. Aliquots of 100 and 200 μl were spread on LB agar plates containing appropriate antibiotics and incubated at 37°C for 16 hr.

6.5.2 Introduction of plasmid DNA into *Agrobacterium tumefaciens*

Plasmid DNA was introduced into *Agrobacterium* by electroporation. Firstly, the *Agrobacterium* was grown in 50-ml YEP broth containing 25 mg/l rifampicin at 28°C with shaking at 250 rpm until A_{600} reached 0.6-0.8 (about 9-10 hr). The cells were collected by centrifugation at 5,000 x g for 5 min at 4°C, gently washed 3 times with 20 ml of 10% (v/v) chilled glycerol, and centrifuged as above. The cells were re-suspended in 125-150 µl of 10% (v/v) chilled glycerol and then used for transformation.

Before transformation, a 0.2-cm electroporate tube was sterilized by immersion in 70% ethanol, washed with sterile distilled water and chilled prior to use. To perform transformation, 2 µl of plasmid DNA (0-5 µg) were mixed with 25 µl of competent cell solution and chilled on ice for 1 min After the sample mixture was loaded into the electroporate tube and the tube was placed in the chamber of an electroporator (BIO-RAD), an exponential pulse of 2,800 V, 25 µF and 400 Ω was applied to the sample. After the addition of 1 ml of YEP medium, the sample was incubated with horizontal shaking at 28°C for 1 hr. Aliquots of 100, 200 and 300 µl were spread on YEP agar containing appropriate antibiotics and incubated at 28°C for 2-3 days.

7. DNA analysis

7.1 Genomic DNA extraction

Genomic DNA was extracted from plant tissue using DNA Sabai Kit, developed by Plant Biochemistry and Molecular Genetics Laboratory, Department of Biotechnology, Faculty of Science, Mahidol University. To extract the DNA, a few young leaves were incubated at 65°C in 500 µl of buffer DA and 40 µl of buffer DB and ground while incubating. The slurry was incubated at 65°C for 10 min and then at room temperature for 1 min. The DNA was extracted with 500 µl of

chloroform/isoamyl alcohol mixture (24:1, v/v) and centrifuged at 10,000 x g for 10 min. The DNA in the aqueous phase was precipitated with 500 µl of ice-cold isopropanol, centrifuged at 7,000 x g for 10 min at 4°C, washed once with ice-cold 80% ethanol and air dried at room temperature. The pellet was re-suspended in 20 µl of sterile deionized water and treated with 10 µg/ml of RNase A at 65°C for 10 min. The DNA was re-precipitated with ethanol, re-suspended in 50 µl of sterile deionized water, and stored at -20°C until used.

7.2 Southern blotting

Southern blot was performed to identify a specific DNA sequence in the genomic DNA sample. The genomic DNA of 40 µg was digested with *Bgl*II and *Nco*I, and then electrohoresed on 0.8% agarose using a constant voltage of 100 V for 6 hr. The gel was submerged with gentle shaking in depurination solution (0.25 M HCl) for 30 min, denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 2x15 min, neutralization solution (0.5 M Tris-HCl; pH 7.0, 1.5 M NaCl) for 2x15 min, and then transferred to a positively charged nylon membrane using downward capillary transfer method (Sambrook *et al*., 1989). Blotting was performed at room temperature for 4 hours. After finished, the damp nylon membrane was carefully removed and the DNA was fixed onto the membrane using GS GENE LINKER™ UV chamber (Bio-Rad, USA).

In this study, Southern hybridization was performed using a fragment of GBSSI inverted repeats, labeled with digoxigenin (DIG), as probe. The DIG-11-dUTP was incorporated into the DNA fragment using PCR DIG probe synthesis kit (Roche). A 50-µl PCR reaction contained 25 µg of pKANNIBAL::35S::GBSSi, 2.6 units of enzyme mix, 0.4 µM of each GBSSi-F1 and GBSSi-R1 primers and 1X PCR DIG probe synthesis mix (200 µM of each dATP, dCTP, and dGTP, 130 µM dTTP, 70 µM DIG-11-dUTP) in 1X PCR buffer (20 mM Tris-HCl; pH 8.4, 50 mM KCl). The PCR program was set up as follows; initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 46°C for 30 sec and extension at 72°C for 30 sec, and , finally, additional extension at 72°C for 10 min. As compared to the unlabeled control, a higher molecular weight shift of the DIG-labeled PCR product could be observed by agarose gel electrophoresis.

To perform Southern hybridization, 5 µl of the denatured DIG probe was added to 10 ml of DIG easy hybridization buffer (Roche). The resulting buffer was incubated with the membrane for overnight at 43°C. After completed, the membrane was sequentially washed for 3x5 min at room temperature in 30 ml of low stringent wash buffer (2X SSC, 0.1% SDS) and for 2x20 min at 68°C in 30 ml of high stringent wash buffer (0.2X SSC, 0.1% SDS). The membrane was equilibrated for 5 min at room temperature in 10 ml wash buffer (Roche) prior to being subjected to immunological signal detection.

To detect the hybridization signals, the membrane was incubated at room temperature for at least 2 hr in 10 ml blocking buffer (Roche) and then for 30 min in antibody solution (blocking buffer containing 75 mU/ml anti-DIG antibody). The membrane was washed for 2x15 min in 15 ml wash buffer (Roche) and equilibrated for 5 min in 20 ml detection buffer (0.1 M Tris-HCl; pH 9.5, 0.1 M NaCl). At this step the membrane was removed from the hybridization tube and placed on a plastic bag with DNA side facing up. Two ml of CDP* solution were added and spread onto the membrane. After incubation at 37°C for 10 min to enhance the signals, the membrane was placed in a film cassette, exposed to an X-ray film (Kodak) for 20 min to 2 hr depending on the signal intensity. To strip the DNA probe, the membrane was thoroughly rinsed in sterile distilled water, washed for 2x15 min at 37°C in stripping buffer (0.2 M NaOH, 0.1% SDS), and 5 min in 2X SSC. The membrane could be kept at 4°C in 2X SSC until used.

8. RNA analysis

8.1 Extraction of RNA from plant tissues

Total RNA was extracted from the *in vitro* plantlets using frozen phenol method with some modifications (Stiekema *et al.*, 1988). Plantlets were ground to a fine powder in liquid nitrogen and mixed with equal volume of extraction buffer (0.2 M NaOAc; pH 5.2, 1% SDS, 0.01 M EDTA; pH 8.0) and phenol saturated with DEPC-H_2O. The mixture was left on the bench until the frozen tissue was thawed and then mixed by vigorous vortexing. After the sample was centrifuged at 14,000 x g for 15

min at room temperature, the upper aqueous phase was re-extracted with 0.5 ml of phenol-chloroform mixture and centrifuged at 14,000 x g for 15 min at 4°C. RNA in the aqueous phase was precipitated with 1/3 volume of 10 M LiCl and incubated at 4°C overnight. The pellet was recovered by centrifugation at 14,000 x g for 15 min at 4°C, washed with 2.5 M LiCl and, subsequently, with 70% ethanol. The RNA pellet was dissolved in 25-30 µl DEPC-H_2O and stored at -80°C until used.

8.2 DNase I treatment

Contamination of genomic DNA in the RNA sample was eliminated by treating the sample with DNaseI. A 50-µl reaction containing 10 µg RNA solution and 2 units of rDNaseI (Ambion) in 1X rDNaseI buffer was incubated at 37°C for 30 min. After the addition of 5 µl of DNase inactivation reagent (Ambion), the sample was centrifuged at 10,000 x g for 1.5 min at 4°C. The supernatant, which contained exclusively the RNA, was carefully transferred to a new tube and stored at -80°C until used.

8.3 Formaldehyde gel electrophoresis

RNA was fractionated under denaturing condition in an agarose-formaldehyde gel. The gel was prepared by dissolving 1.2% agarose in 1X MOPS running buffer (20 mM MOPS; pH7.0, 8 mM NaOAc, 1 mM EDTA; pH 8.0). After the agarose solution was allowed to cool to 60°C, formaldehyde at a final concentration of 2.2 mM was added and the mixture was then poured into a casted tray. To denature the RNA, 1 µg RNA sample was mixed with 10 µl of RNA loading dye (50% deionized formamide, 6.142% formaldehyde, 1X MOPS, 0.05% bromophenol blue 0.2 mg/ml ethedium bromide, 10% glycerol), heated at 65^{o}C for 10 min and quickly chilled on iced for 1 min. Electrophoresis was performed in 1X MOPS buffer for 3 hr at a constant voltage of 50 V. The RNA was visualized on a UV transilluminator and photographed using a gel documentation system (GENE GENIOUS Bio Imaging equipped with GeneSnap software, SYNGENE).

8.4 Semi-quantitative RT-PCR

8.4.1 First-strand cDNA synthesis

First-strand cDNA synthesis reaction was carried out using SuperScript^{TM}III reverse transcriptase (Invitrogen). Firstly, a 13-µl reaction mixture containing 1 µg total RNA, 50 µM of oligo$(dT)_{20}$, and 10 µM of each dNTP was incubated at 65^{o}C for 5 min and immediately cooled on ice for 5 min. Secondly, a mixture of 200 units of SuperScript^{TM}III reverse transcriptase, 40 units of RNase^{TM} Out (Recombinant Ribonuclease Inhibitor) and 10 mM DTT in 1X First-strand buffer (20 mM Tris-HCl; pH 8.4, 50 mM KCl) was added to the reaction mixture followed with incubation at 50^{o}C for 60 min. Finally, the reaction was terminated by heating at 70^{o}C for 15 min.

8.4.2 PCR amplification

Semi-quantitative RT-PCR reaction contained two different pair of primers, one of which amplifies the gene of interest while the other amplifies a fragment of internal standard. In this study, a 275-bp fragment of GBSSI transcript was determined, using GBSSi-F1 and GBSSi-R1 primers (Table 3.3), along with the presence of a 400-bp fragment of *MeEF1*-α as an internal standard. The primers used to amplify the *MeEF1*-α cDNA fragment were EF-F (5′-TCCAATCTGACCAGGGTGG-3′) and EF-R (5′-GTATGCCTGGGTGCTTGACA-3′) primers. A 15-µl PCR reaction contained 20 ng cDNA template, 0.4 mM dNTPs, 0.4 µM of each primer and 0.6 U of *Taq* DNA polymerase (Fermentas) in 1X *Taq* buffer containing 1.5 mM $MgCl_2$. The PCR was performed as followed: 94^{o}C for 2 min, 35 cycles of 94^{o}C for 30 sec, 60^{o}C for 30 sec and 72^{o}C for 30 sec followed by additional extension at 72^{o}C for 10 min.

9. Histochemical analysis

In the presence of X-Gluc, the histochemical staining of GUS would proceed through an unstable intermediate, which then undergoes oxidative dimerization to yield a deep blue product of ClBr-indigo. In this study, histochemical analysis was

performed using a method described by Jefferson *et al*. (1987). The reagent mixture typically contained three components: the substrate (X-Gluc), the buffer (0.1 M sodium phosphate; pH 7.0) and the oxidation catalyst (0.5 mM of each potassium ferric- and ferrocyanide; pH 7.0 and plus 10 mM EDTA; pH 7.0) (Table 3.4). It should be noted that X-Gluc is fairly unstable. Therefore, a 0.02 M stock solution in N, N' dimethylformamide (DMF) needs to be prepared every 6 months, aliquoted and kept in the dark at -20°C. Additionally, the ferrous ion in ferrocyanide could undergo oxidation yielding ferric ion, ferric- and ferrocyanide stock solutions must be prepared every 2 months; otherwise, a change of color from pale to deep yellow could be observed.

Table 3.4 List of ingredients in the reagent mix for histochemical analysis

Stock solution	Final concentration	Amount (µl)
1.0 M Sodium phosphate 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
Distilled water		600
Total volume		1000

To perform histochemical analysis, the plant tissues were incubated in the reagent mix at 37°C for 1 hr to overnight until the desired staining intensity was obtained. Interference by chlorophyll pigments was minimized by washing the stained tissues several times with 70% ethanol.

10. Iodine staining assay

Free-hand cross-sections of transgenic and wild-type plant grown in sterile culture were obtained. Starch granules in the plant tissues were stained with iodine

solution (Lugol's reagent; 5% I_2 and 10% KI) and visualized using Olympus microscope model B51 and DP71. In this study, four different levels of the iodine-stained starch granules were observed, thus indicating different level of GBSSI gene silencing. Transformants whose granules were stained blue with iodine were classified as non GBSS-silencing lines since their amylose/amylopection ratio was not significantly altered. Transformants carrying granules with a large blue core and a small red outer layer were classified as weak silencing lines. Transformants with granules having a small blue core and a large red outer layer were classified as strong silencing lines. Lastly, transformants with granules of varying size of the blue cores were classified as intermediate silencing lines.

11. Investigation of the effect of antibiotics on *Agrobacterium* growth suppression

In order to study the resistance of *A. tumefaciens* to antibiotics used in transformation experiments, *Agrobacterium* strains AGL1, LBA4404 and EHA105 harboring pCAMBIA1301 were grown on YEP medium containing 50 mg/l rifampicin and 0-100 mg/l kanamycin, 0-100 mg/l hygromycin B, 0-500 mg/l carbenicillin, 0-500 mg/l cefotaxime, or 0-500 mg/l augmentin. After the culture was incubated at 28ºC for 2 days, the number of bacterial colonies appeared on each culture plate was recorded. These experiments were repeated 3 times and each of which was carried out using 3 replica plates.

12. Investigation of the effect of antibiotics on cassava shoot regeneration

According to the Topic 3.3, which described the induction of shoot or plantlets via organogenesis, various concentrations of antibiotics, including 0-100 mg/l kanamycin, 0-100 mg/l hygromycin, 0-500 mg/l carbenicillin, 0-500 mg/l cefotaxime, or 0-500 mg/l augmentin, were tested to investigate the effects on shoot regeneration efficiency. The explants were transferred to new medium every week and emerging of the regenerated shoots was observed for 4 weeks. These experiments were repeated 3 times and there were 3 replicas for each treatment.

13. Preparation of pre- and post-plasmolysed tissue

Before being subjected to transformation, the somatic embryo-derived cotyledons used as explants must undergo the pre-plasmolysed stage. To perform this, the explants were placed, with their abaxial side down, onto cassava transformation medium (CTM) and incubated in the dark at 26°C for 20 hr. After particle bombardment, the post-plasmolysis was performed using the same condition as pre-plasmolysis before transferring the transformed explants to COM medium.

14. *Agrobacterium* transformation

14.1 Transformation of cassava with *Agrobacterium*

A colony of *Agrobacterium* strain EHA105 or LBA4404 carrying a binary vector (Figure 3.1) was inoculated to 10 ml YEP liquid medium and incubated at 28°C until the OD_{600} reached 1.2. The bacterial culture was diluted 1/50 with CBM supplemented with 100 µm acetosyringrone and 0.5% glucose and used in co-cultivation with cassava explants. The co-cultivation was performed in the dark at 28°C for 48 hr on shoot organogenesis induction medium (COM). Selection and regeneration of transformed cassava plants were described in the Topic 16.1.

14.2 Transformation of potato with *Agrobacterium*

Agrobacterium strain LBA4404 carrying a binary vector was used in potato transformation using the protocol described by (Banerjee *et al.*, 2006) with some modifications. Firstly, the *Agrobacterium* was grown in 3-ml YEP medium supplemented with appropriate amount of antibiotic at 28°C with shaking at 250 rpm until the OD_{600} reaching 1.0–1.2. To use in potato transformation, the culture was diluted with 15-ml YEP medium devoid of any antibiotics. The potato inter-node cuttings obtained from healthy stems of 4 weeks old were used as explants. Approximately 20 node segments were placed on a plate containing 20 ml of liquid PBM medium (MS medium with 2% sucrose; pH 5.8) devoid of any phytohormones.

After the addition of 100-µl diluted *Agrobacterium* culture, the plate containing explants was shaken on a platform shaker at 50 rpm for 30 min at room temperature, sealed and incubated in the dark for 48 hr. After 2 days of co-cultivation, the node segments were washed for 2x30 min with sterile distilled water, blotted dry on tissue paper and then transferred to PBM agar plate supplemented with 10 mg/l hygromycin B.

15. Biolistic transformation

Biolistic transformation was carried out using the biolistic PDS-1000/He system (Bio-Rad) (Figure 3.11). With high pressure helium, released by a rupture disk, a macrocarrier sheet loaded with millions of microscopic tungsten or gold microcarriers is propelled toward target cells at high velocity. To coat the microcarriers with DNA, a protocol described by Sanford *et al.* (1993) was followed. To prepare the microcarriers, 60 mg of 1-µm gold microparticles were washed with 1 ml of freshly prepared 70% ethanol vigorously mixed for 3-5 min in the presence of ethanol and then incubated for 15 min. After a quick spin for 5 sec, the pellet was recovered and washed for 3x1 min in 1 ml sterile water followed with a quick spin for 2 sec. Finally, 1 ml of 50% glycerol was added to bring the microparticle to a final concentration of 60 mg/ml. To be coated with DNA, 30-mg gold particles were successively mixed with 5 µl of plasmid DNA (1 µg/µl), 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine (free base). The mixture was continuously vortexed for 2-3 min, allowed to settle for 1 min and spun down for 2 sec. The microcarrier pellet was washed with 140 µl of 70% ethanol, spun down for 2 sec and then re-suspended in 48 µl of 100% ethanol. At this step, mixing of the resuspended microcarriers could be done by gently flicking the tube or by vortexing at low speed for 2-3 sec.

Prior to bombardment, a microcarrier launch assembly, a macrocarrier, a rupture disk retaining cap and stopping screens were sterilized by autoclaving at 121°C for 15 min. An 8-µl aliquot of the DNA-coated gold microcarriers was loaded and spread evenly in the center of the macrocarrier (approximately 1 cm in diameter) using a pipette tip. The ethanol used to resuspend the microcarriers was allowed to evaporate

(approximately 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 at 200 psi above the selected rupture disk burst pressure. In this experiment, the regulator was set at 1,550 psi because the bombardment was performed with a 1,350 psi rupture disk. Assemble of the bombardment chamber was carried out according to the instructions by manufacturer (Figure 3.12). After a petridish containing pre-plasmolysed explants, as described in Topic 13, was placed on the target shelf at the target distance of 9 cm, bombardment was performed using the conditions listed in Table 3.5.

Table 3.5 Conditions for particle bombardment

Parameter	Tested Conditions
1. Plasmid DNA	1 μg/μl
2. Gold particle size	0.6 μm
3. Acceleration pressure	1,350 psi
4. Target distance	9 cm
5. Chamber vacuum pressure	28 inches Hg
6. Number of bombardment	1

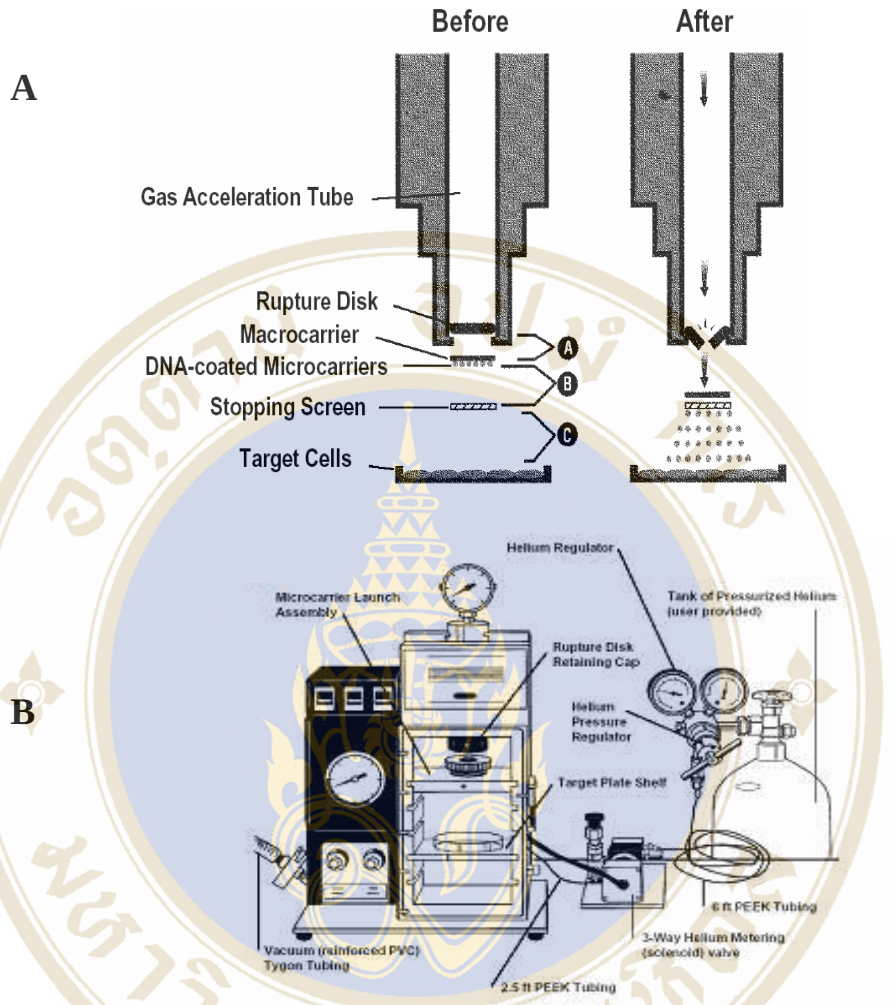


Figure 3.11 Biolistic transformation system

A. 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. **B.** Components for the PDS-1000/He Particle Delivery System (Bio-Rad).

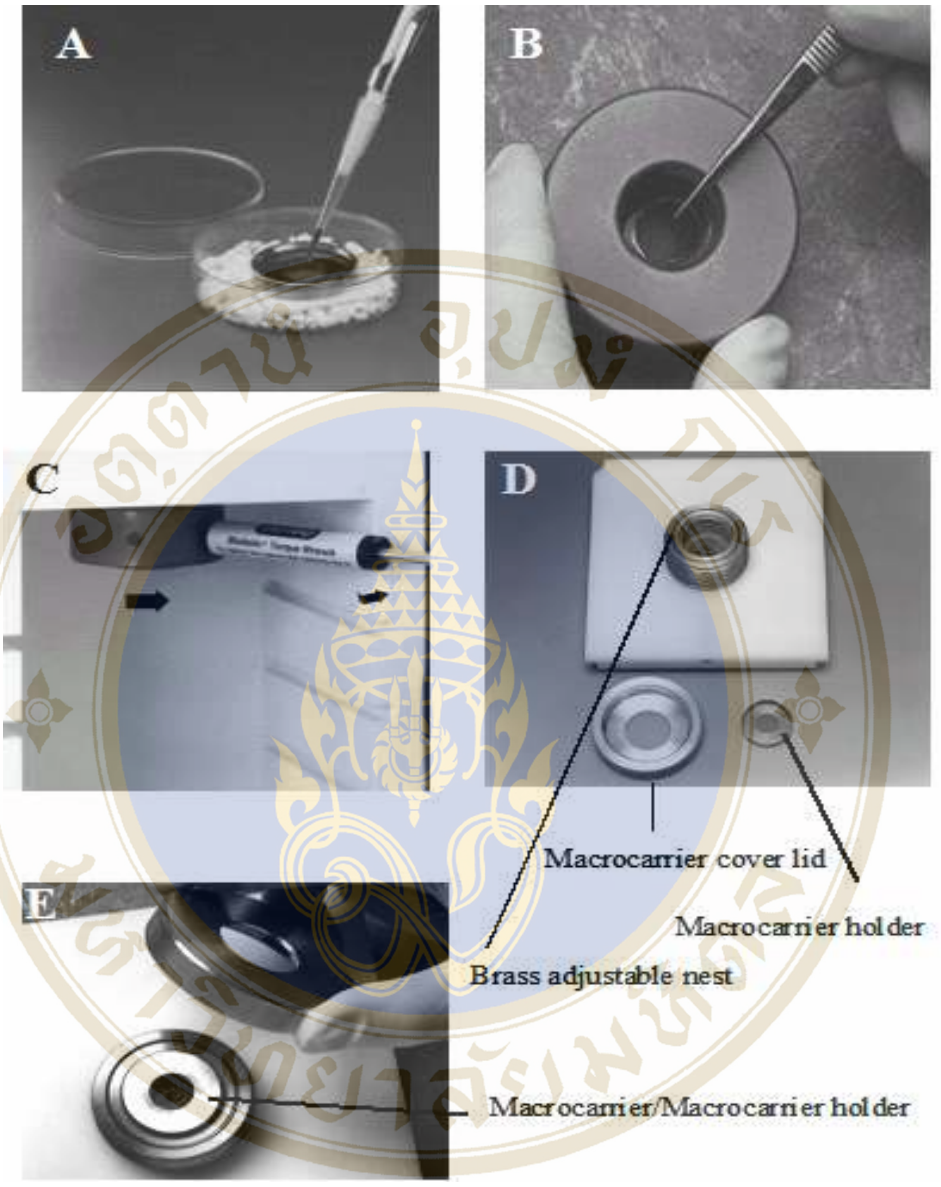


Figure 3.12 Steps of bombardment

A. Loading of DNA-coated microcarriers onto a macrocarrier/macrocarrier holder. **B.** Insertion of a rupture disk insertion into recess of retaining cap. **C.** Application of proper torque to retaining cap with a torque wrench. **D.** Removal/replacement of macrocarrier coverlid with assembled fixed nest. **E.** Placing of macrocarrier holder (with macrocarriers properly inserted) on top of the fixed nest of macrocarrier launch assembly.

16. Selection and regeneration of the transgenic plants

16.1 Cassava

After co-cultivation with *Agrobacterium*, the cassava explants were washed twice with sterile distilled water and once with liquid COM supplemented with 300 mg/l cefotaxime and blotted dry on filter paper. The explants were cultured in the dark at 26°C on COM with 300 mg/l cefotaxime (for suppression of further bacterial growth) with a regular transfer to new medium every week. As for transient expression analysis, the explants were harvested and subjected to histochemical GUS activity assay. After 4-6 weeks of *Agrobacterium* infection, the shoot primordial emerging from the explants was detached and transferred to CEM. After 4 weeks, the number of elongated shoots was recorded and then transferred to CRM.

After bombardment, the explants were kept in the dark at 26°C for 24 hr for post-plasmolysis, transferred to COM without selective agent for 7 days, and then to new COM medium containing selective agent every week. As for transient expression analysis, the explants were harvested and subject to histochemical GUS activity assay. After 4-6 weeks of bombardment, the shoot primordial emerged from the explants was detached and transferred to CEM containing selective agent. After 4 weeks, the number of elongated shoots was recorded, and then transferred to CRM.

Healthy cassava plants were further subjected to molecular analyses including PCR, RT-PCR and Southern blot hybridization. The *in vitro* storage roots from selected transgenic lines of cassava were used for starch composition analysis by iodine staining. In addition, secondary growth of the root was anatomically examined by staining of the root cross section with safranin O.

16.2 Potato and its mini-tuber induction

After 2 days of co-cultivation, the node segments were washed for 2x30 min with sterile distilled water, blotted dry on tissue paper and then transferred to PBM medium supplemented with 10 mg/l hygromycin B. The explants were cultured on selective medium until shoots were regenerated from the nodes. After the elongated

shoots primordial were approximately 2-3 cm long, they were cut and transferred to PBM agar bottles. As for transient expression analysis, the explants were harvested and subjected to histochemical GUS activity assay. Transgenic potato plants were cultured under light condition for 4 weeks prior to being subjected to molecular analyses including PCR and RT-PCR. Mini tubers were induced by cultivation of node segments on PTM medium (MS medium, 8% sucrose, 5 mg/l BAP; pH 5.8 and solidified with 6 g/l bacto agar). After incubation in the light at 26°C for 1 week, the stem segments were transferred to the dark for 4 to 6 weeks. The mini-tubers developed from the shoots were further subjected to iodine staining.

16.3 Tobacco

Bombarded tobacco leaves that had been incubated overnight were cut into 1 cm^2. The cut leaves were placed on selective medium agar plates (RMOP supplemented with 20 mg/l hygromycin B; see Appendix B). After 3 to 4 weeks, the green shoots emerged from the cut leaves observed were then transferred to MS agar medium bottles. The transformed shoots were cultured until mature to become whole plants, which generally take about 4 weeks. Further analyses including PCR, RT-PCR, GUS activity assay and iodine staining were carried out to confirm the presence of transgenes in the tobacco transgenic lines.

CHAPTER IV
RESULTS

PART I Cassava tissue culture and transformation system

1. Effect of antibiotics on *Agrobacterium* growth suppression

For transformation system using *Agrobacterium*, the amount of antibiotic used to eliminate the excess cell on the explants is very important. The effects of kanamycin, hygromycin B, carbenicillin, cefotaxime, and augmentin on *Agrobacterium* growth suppression on COM medium were tested. The *Agrobacterium* strains used in this study included the wild-type strains (LBA4404 and EHA105) and the strain harboring plasmid vectors (AGL1 harboring pCAMBIA1301, EHA105 harboring pCAMBIA1301 and LBA4404 harboring pCAMBIA1301::p54::*gus*). These *Agrobacterium* strains were grown at 28°C for 2 days on shoot organogenesis induction medium (COM) supplemented with 50 mg/l rifampicin and various concentrations of cefotaxime, augmentin, carbenicillin, kanamycin, and hygromycin B. According to the results shown in Table 4.1, 300 mg/l of cefotaxime could be used to suppress the growth of these *Agrobacterium* strains. Therefore, cefotaxime at 300 mg/l would be used in experiments involving *Agrobacterium*-mediated transformation.

Table 4.1 Effect of antibiotics on *Agrobacterium* growth suppression

Antibiotic	Concentration (µg/ml)	Viability of *Agrobacterium* *				
		LBA4404	EHA105	AGL1	EHA105	LBA4404
				pCAMBIA 1301	pCAMBIA 1301	pCAMBIA 1301::p54::*gus*
No antibiotic	0	+++	+++	+++	+++	+++
Cefotaxime	200	-	+	+	+	-
	300	-	+	+	-	-
	400	-	+	+	-	-
Augmentin	25	+++	+++	+++	++	+
	50	+++	+++	+++	++	+
	75	+++	+++	+++	++	+
	100	+	+	+++	+	+
Carbenicillin	200	+++	+	+++	+	+
	300	+++	+	+++	+	+
	400	+++	+	+++	+	+
Kanamycin	25	ND	+	ND	+++	ND
	50	ND	-	ND	+++	ND
	100	ND	-	ND	++	ND
Hygromycin B	25	ND	-	ND	+++	ND
	50	ND	-	ND	++	ND
	100	ND	-	ND	+	ND

*ND: not determined

\+ : degree of relative viability of *Agrobacterium*

\- : no growth of *Agrobacterium*

2. Effect of antibiotics on shoot organogenesis of cassava

The effect of antibiotics on cassava shoot organogenesis was studied on COM supplemented with various antibiotics. To induce shoots or plantlets via organogenesis, the culture was kept in dim light at 26°C and regularly transferred to new COM medium containing the same antibiotic. Viability of the regenerated shoots was observed at the 4th week.

Based on the results obtained, the Hanatee cultivar was found to withstand the antibiotics at slightly higher concentration than KU50 (Table 4.2). The carbenicillin, augmentin and cefotaxime were toxic to both cultivars, as seen from the reduction in shoot organogenesis induction frequency from 90% to <30%. Even cefotaxime slightly affected shoot organogenesis but it is cheaper than carbenicillin and augmentin and at the concentration of 300 mg/l, it can be used to inhibit the growth of *Agrobacterium* (Table 4.1). Therefore, cefotaxime at 300 mg/l would be used in culture medium of somatic cotyledons after *Agrobacterium* infection.

Kanamycin at concentration higher than 50 mg/l could completely inhibit shoot organogenesis in both cassava cultivars (Table 4.2). By combining kanamycin at 15 to 25 mg/l with cefotaxime at 300 mg/l, *Agrobacterium* would be killed while the transformed cells that obtained kanamycin resistance gene from pKANNIBAL could be selected (Table 4.3). Hygromycin B at 50 mg/l could completely inhibit shoot organogenesis in both cultivars (Table 4.2). By combining hygromycin B at 10 to 20 mg/l with cefotaxime at 300 mg/l, *Agrobacterium* would be killed while the transformed cells that obtain hygromycin resistance gene from pCAMBIA1301 would be selected (Table 4.3). Since the shoot organogenesis induction frequency was extremely low in the presence of kanamycin or hygromycin B, both antibiotics should not be added into selective medium.

Table 4.2 Effect of antibiotics on shoot organogenesis of cassava cultivars KU50 and Hanatee

Antibiotic	Concentration (mg/l)	Shoot organogenesis induction frequency (%)*	
		KU50	Hanatee
No antibiotic	0	90 ± 3	87 ± 2
Hygromycin B	25	5 ± 1	8 ± 1
	50	0	0
	100	0	0
Kanamycin	25	12 ± 2	14 ± 1
	50	2 ± 1	1 ± 1
	100	0	0
Carbenicillin	100	64 ± 2	74 ± 3
	250	46 ± 2	58 ± 1
	350	37 ± 1	59 ± 2
	500	30 ± 3	58 ± 2
Cefotaxime	100	62 ± 2	68 ± 1
	250	45 ± 4	46 ± 2
	350	31 ± 3	33 ± 4
	500	24 ± 4	26 ± 3
Augmentin	100	67 ± 2	76 ± 1
	250	48 ± 3	61 ± 2
	350	39 ± 1	61 ± 3
	500	33 ± 1	58 ± 2

* The experiment was repeated 3 times comprising of 50 explants per treatment

Table 4.3 Combining effect of antibiotics on shoot organogenesis of cassava cultivars KU50 and Hanatee

Antibiotic	Concentration (mg/l)	Shoot organogenesis induction frequency (%)*	
		KU50	Hanatee
Cefotaxime 300 mg/l + Kanamycin	5	35 ± 3	51 ± 4
	10	28 ± 1	32 ± 3
	15	21 ± 1	28 ± 1
	20	18 ± 1	21 ± 2
	25	12 ± 2	14 ± 1
	30	2 ± 1	5 ± 1
	40	0	0
	50	0	0
Cefotaxime 300 mg/l + Hygromycin B	5	29 ± 2	45 ± 3
	10	18 ± 1	24 ± 2
	15	15 ± 2	22 ± 2
	20	11 ± 1	17 ± 1
	25	5 ± 1	8 ± 1
	30	1 ± 1	3 ± 1
	40	0	0
	50	0	0

* The experiment was repeated 3 times comprising of 50 explants per treatment

3. Investigation of the effect of acetosyringone on viability of *Agrobacterium*

Acetosyringone (4'-Hydroxy-3,5-dimethoxyacetophenone) is a phenolic compound that acts as expression inducer of the virulence (*vir*) genes in *Agrobacterium tumefaciens*. *A. tumefaciens* detects specific compounds such as acetosyringone in plant wounds, thus resulting in the expression of *vir* genes present on the Ti plasmids. Expression of the *vir* genes leads to the transfer of a particular region on the Ti plasmids, called transferred T-DNA, to plant cells. This bacterial DNA becomes stably integrated into nuclear genome of the plant cells. In this study, an enhanced effect of acetosyringone on the growth of *A. tumefaciens* strain EHA105 and AGL1 was observed (Table 4.4). In addition, the growth of these *Agrobacterium* strains was increased when co-cultivation with somatic cotyledons on COM medium containing acetosyringone. The results therefore confirmed that the growth of *Agrobacterium* strain EHA105 and AGL1 required the presence of acetosyringone.

Table 4.4 Effect of acetosyringone on viability of *Agrobacterium*

***Agrobacterium* strain**	**Addition of 100 µM acetosyringone**	**Viability of *Agrobacterium****	
		***Agrobacterium* alone**	**Co-cultivation with cotyledons**
EHA105	Yes	+	+++
	No	-	-
AGL1	Yes	+	++
	No	+	-

* + : degree of relative viability of *Agrobacterium*

- : no growth of *Agrobacterium*

4. Induction of *in vitro* storage root formation in cassava

Although a cassava tissue culture system has successfully been established, little information on the induction of *in vitro* storage roots is currently available. In this study, a method for the induction of cassava *in vitro* storage root reported by Medina *et al.* (2007) was followed using uninodal segments dissected from *in vitro* cassava plants cultivar KU50 as starting materials. The nodes were cultured at 26°C under a 16/8 h photoperiod rhythm of 3,000 lux on storage root formation medium (MS containing 5% sucrose, 0.75% agar, 0.54 µM NAA and 0.44 µM BAP; pH 5.8). The storage root-like structure that is typically larger in diameter and shorter in length than the adventitious or fibrous root was observed within 1-3 months (Figure 4.1). A cross section of this storage root-like structure was stained with safranin O and observed using a light microscope. The secondary tissues that function mainly in starch storage were stain red (Figure 4.1G and 4.1H). In addition, the presence of starch within the storage root-like structure was observed by staining the cross section with iodine (Figure 4.1G and 4.1H).

5. Establishment of a cassava tissue culture system using somatic callus culture

Another cassava tissue culture system that involved the production of somatic cell suspension culture was also developed for the Thai cassava cultivar KU50. The friable callus was cultured in MS liquid medium containing 3% sucrose, 1 mg/l 2,4D, 1 mg/l BAP and 100 mg/l *myo*-inositol; pH 5.8. The suspension culture was kept at 26°C under a 16/8 photoperiod with shaking at 110 rpm. After 2 months, the young callus was further developed to form globular callus and cluster callus. The green mature calli were dissected and stained with iodine to investigate the presence and accumulation of starch. The globular-like structure and cluster calli were observed to accumulate starch at different amount. Although, both kinds of calli were green, the globular callus was observed to store starch at a significantly smaller amount (Figure 4.2). In addition, from morphological investigation of the cross section, the globular callus appeared to be composed of homogenous cells, while the cluster callus possibly resulted from aggregation of small immature callus. The observation of starch in these

calli suggested that callus culture in liquid medium could be used as an alternative system to study starch biosynthesis in cassava and this system would be less time consuming when compared with the system that uses somatic cotyledon as explants.

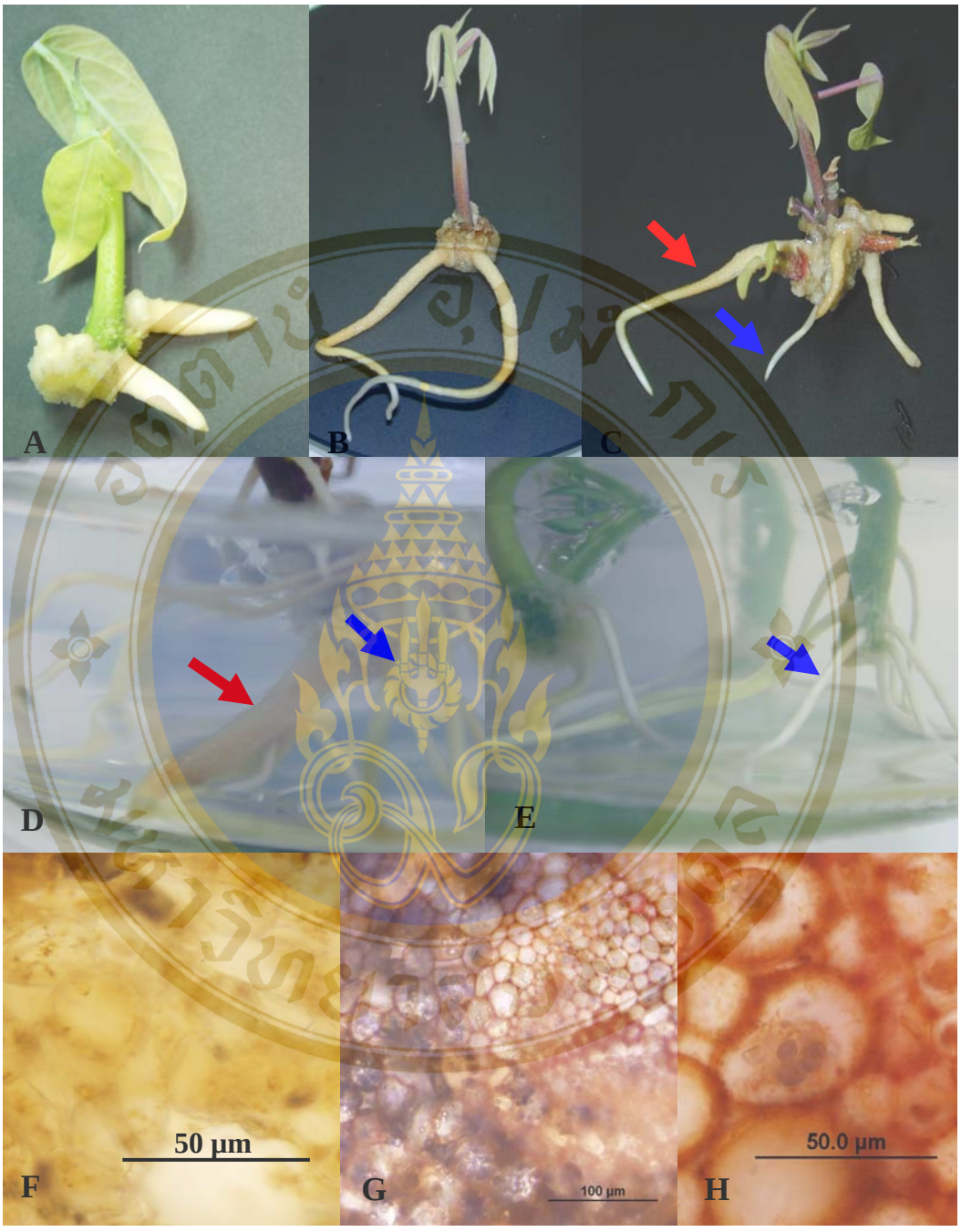


Figure 4.1 Morphological and anatomical characteristics of fibrous and storage roots obtained from *in vitro*

(A-B) Explant with sessile storage roots; (C-D) Explant with fibrous (blue arrow) and storage roots (red arrow); (E) Fibrous roots; Transverse section of fibrous root (F) and storage root (G-H) stained with safranin O and Lugol's reagent.

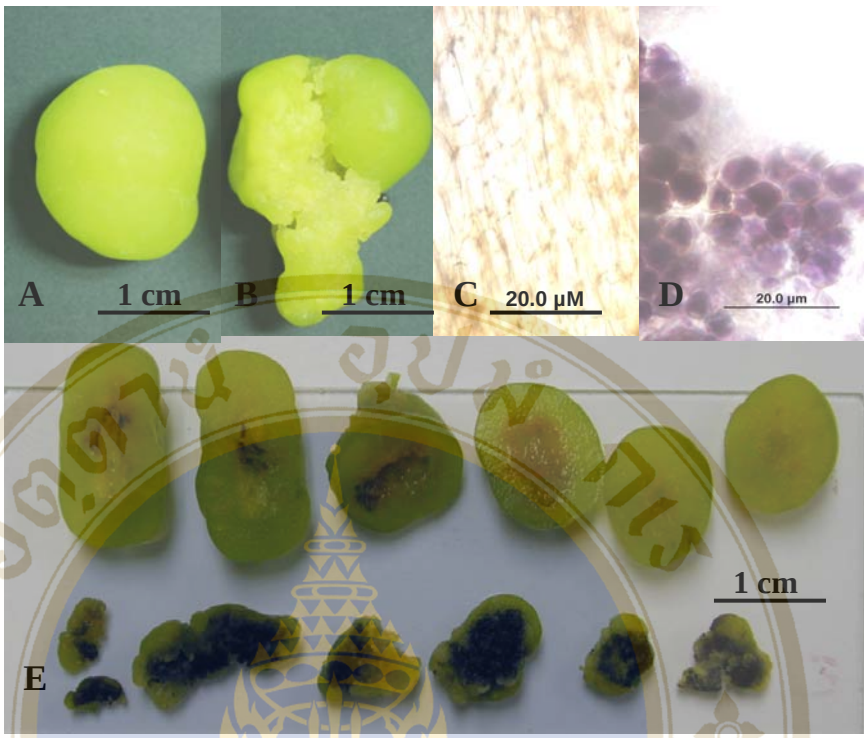


Figure 4.2 Morphology and iodine staining of somatic callus

(A) Globular-like structure; (B) Cluster callus; (C) globular-like structure and (D) cluster callus stained with Lugol's reagent; (E) Cross section of iodine staining of globular-like structure (upper row) and cluster callus (lower row).

PART II Suppression of cassava GBSSI gene by RNA interference

1. Biolistic-mediated transformation

To investigate the effect of GBSSI gene suppression, RNA interference (RNAi) technology was utilized. The GBSSI-RNAi expression cassette (GBSSi) was construct by integrating inverted repeat fragments of cassava GBSSI cDNA into pKANNIBAL vector, thus yielding the plasmid vector pKANNIBAL::35S::GBSSi (Figure 3.5) that would be transformed into cassava. In this experiment, only a fragment containing essential elements for GBSSi expression would be transformed into cassava using biolistic-mediated transformation method. According to the restriction map of pKANNIBAL::35S::GBSSi, *Not*I was considered the most suitable enzyme. *Not*I digestion of pKANNIBAL::35S::GBSSi would yield 2 digested products; 2.9 kb of pKANNIBAL and 3.6 kb of GBSSi containing CaMV 35S promoter, inverted repeat fragment of cassava GBSSI cDNA franking an intron from pyruvate orthophosphate dikinase gene, and an octopine synthase terminator. The GBSSi fragment was coated onto the gold particles and used in cassava transformation via particle bombardment. The plant material used in this study was somatic cotyledon at 14-28 days old. After 4 weeks of bombardment, 50 shoot primordial were transferred to CEM medium for stem elongation and then to CBM medium for root formation. The cassava shoots from different putative lines were collected and then subjected to DNA extraction and PCR analysis. Since the GBSSi fragment contained no marker gene, the transgenic cassava lines obtained in this study were considered marker free.

2. Molecular and transgene integration analysis

2.1 PCR analysis

Integration of transgenes in 50 lines of transgenic cassava was examined by PCR using 2 primer pairs: A+B and C+D (Figure 3.8)**.** The PCR product amplified by primer A+B was observed in 20 out of 50 transgenic cassava lines (Figure 4.3). These

20 lines were then subjected to PCR using primer C+D (Figure 4.4). These were to confirm that all the putative lines contained both fragments of GBSSI inverted repeats. Based on PCR screening of transgenic cassava, the line no.10, 15 and 50 were selected for further analysis.

2.2 Southern hybridization

To determine whether GBSSi fragment was incorporated in cassava genome, Southern analysis was performed using DIG-labeled GBSSI DNA probes. To incorporate DIG-dUTP into the GBSSI DNA fragment, PCR was performed using primer A & B. As compared to the unlabeled control, a higher molecular weight shift of the DIG-labeled probe was observed, thus indicating a successful incorporation of the DIG-11-dUTP into the GBSSI DNA fragment (Figure 4.5).

To perform Southern analysis, genomic DNA extracted from wild type and transgenic cassava lines no.10, 15 and 50 was double digested with *Bgl*II and *Nco*I. A single band observed in wild type should represent an endogenous GBSSI gene, while on additional band in the strong silencing line no.50 and 2 extra bands in the weak silencing line no.15 and the non-silencing line no.10 should represent the GBSSi transgene used in cassava transformation (Figure 4.6).

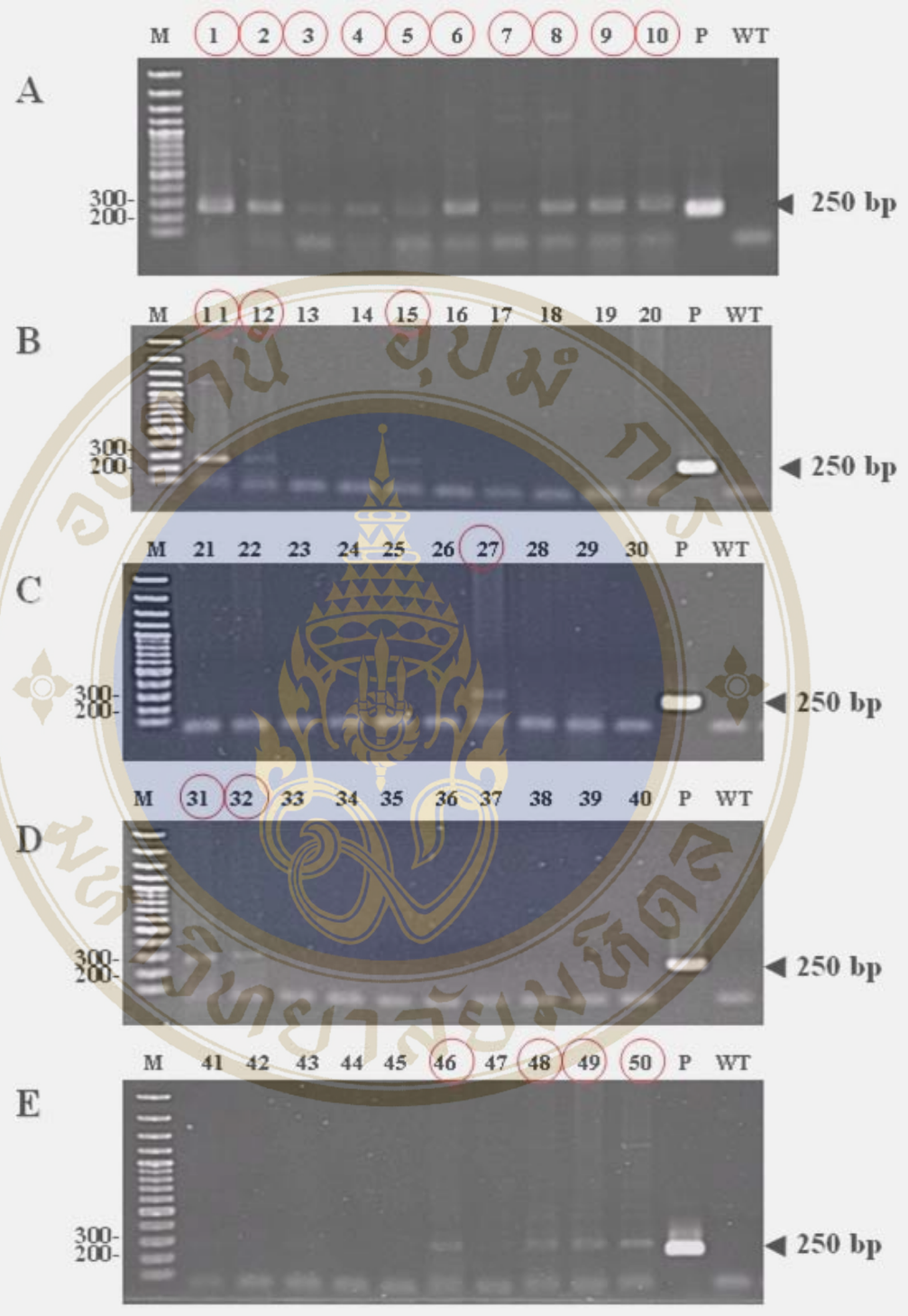


Figure 4.3 Analysis of putative transgenic cassava plants by PCR using primer A+B. Lanes 1-50, particular lines of transgenic cassava bombarded with GBSSi fragment; P, pKANNIBAL::35S::GBSSi control; WT, wild-type cassava cultivar KU50; M, 100-bp DNA Ladder Plus (Fermentas). Red circle indicated the line with expected PCR product.

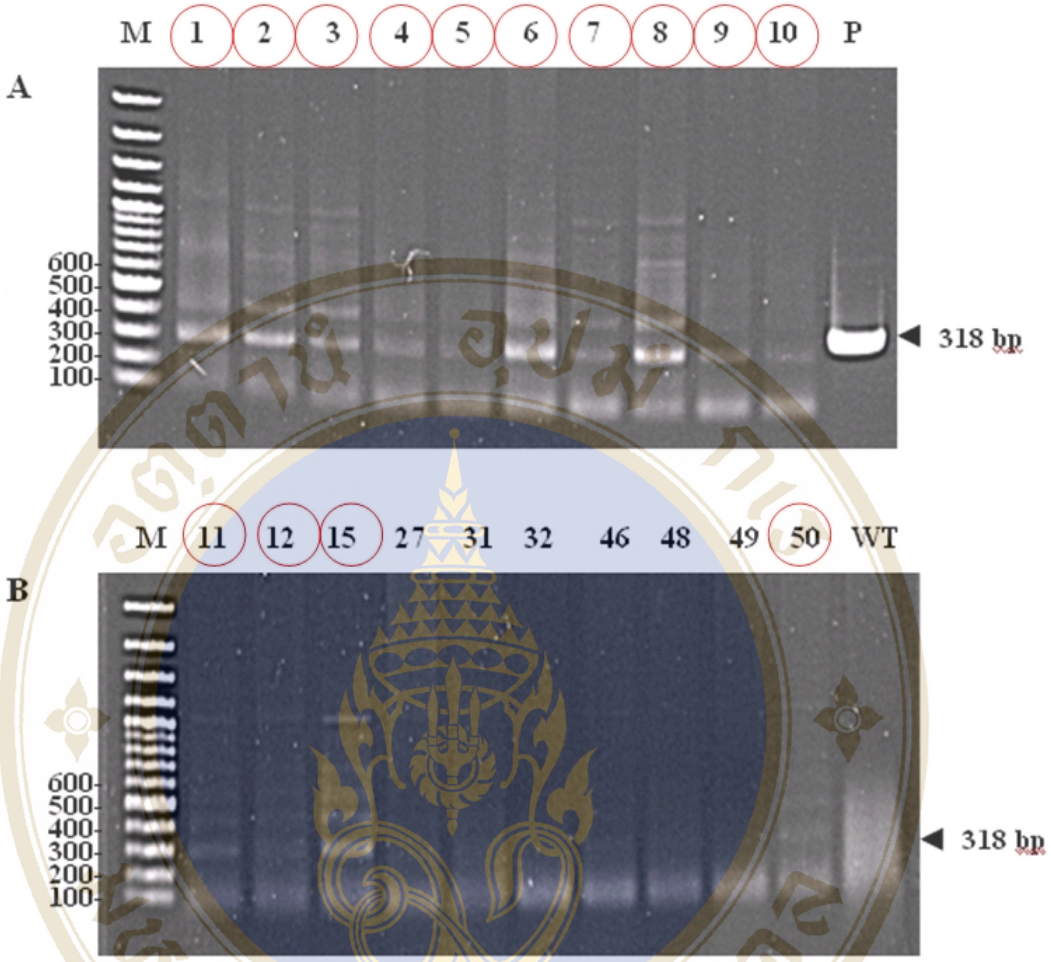


Figure 4.4 Analysis of putative transgenic cassava plants by PCR using primer C+D. Lanes 1-10, 11, 12, 15, 27, 31, 32, 46, 48, 49 and 50, particular lines of transgenic cassava bombarded with GBSSi fragment; P, pKANNIBAL::35S::GBSSi control; WT, wild-type cassava cultivar KU50; M, 100-bp DNA Ladder Plus (Fermentas). Red circle indicated the line with expected PCR product.

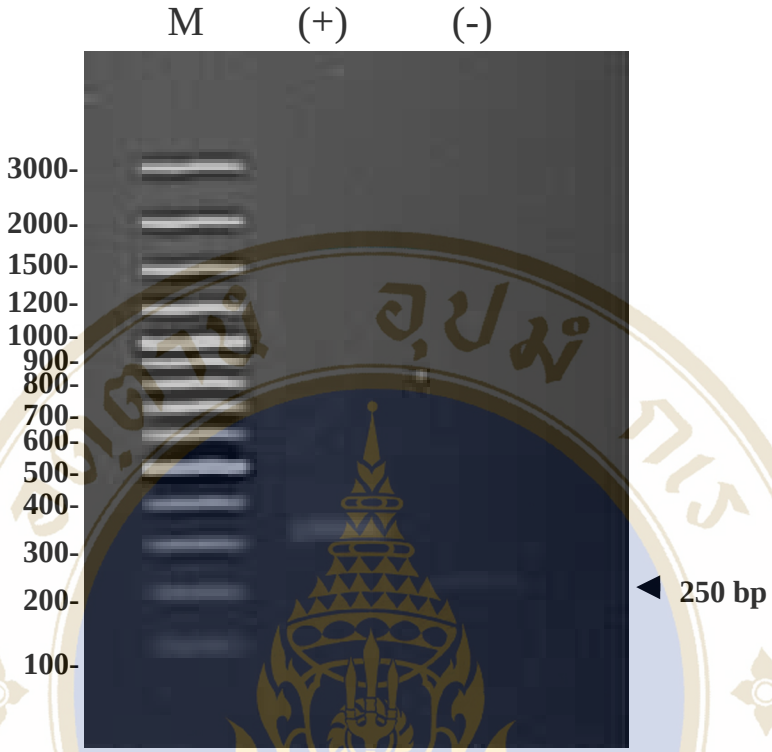


Figure 4.5 Incorporation of DIG into the GBSSI DNA fragment by PCR

PCR was performed in the presence (+) and absence (-) of the DIG-11-dUTP. Sizes of the molecular weight marker (M) were indicated on the left.

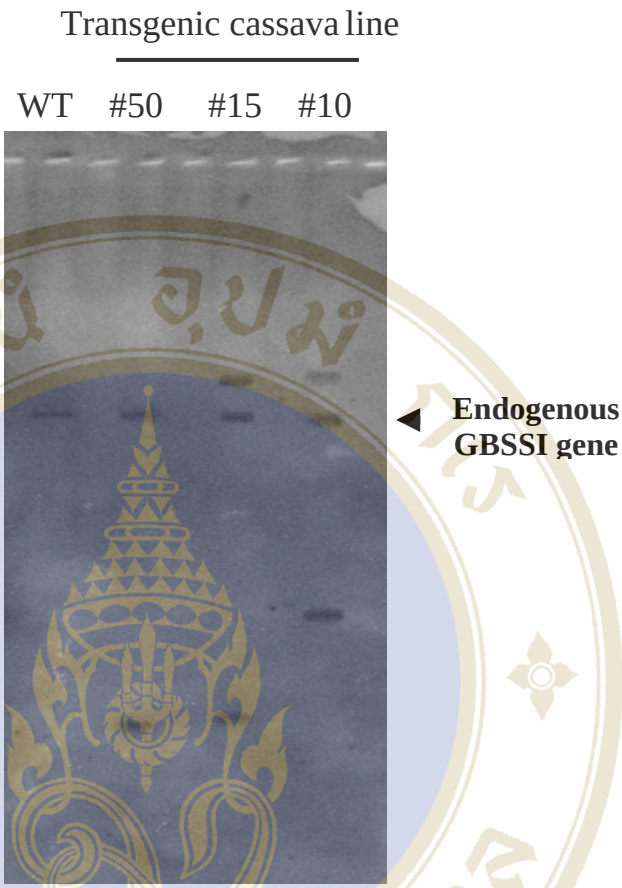


Figure 4.6 Southern hybridization of wild-type and transgenic cassava lines

The *Bgl*II/*Nco*I digested-cassava genomic DNA (40 µg each) was electrophoresed on 0.8% agarose gel, transferred to nylon membrane and probed with a DIG-labeled GBSSI DNA fragment. The signals obtained from the transgenic cassava lines #10, #15 and #50 were compared with those from wild-type cassava cultivar KU50 (WT).

2.3 Determination of GBSSI gene expression by RT-PCR

RT-PCR was performed to determine whether the expression level of GBSSI gene in the cassava transgenic lines would be altered. Quality of the RNA extracted from transgenic lines no.10, 15 and 50 was verified by denaturing gel electrophoresis (Figure 4.7). To ensure equal amount of RNA from different samples, RT-PCR amplification of an elongation factor (*MeEF1-α*) was used as internal control (Figure 4.8B). For GBSSI, RT-PCR was performed for 35 cycles using the primer GBSSi-F1 and GBSSi-R1 (Figure 3.8). According to Figure 4.8A, strong silencing of GBSSI, indicated as the presence of very faint band of GBSSI cDNA in the line no.50. A weak silencing of GBSSI gene was observed in the line no.15, whereas the transcript at normal level was detected in transgenic line no.10.

Figure 4.7 Electrophoresis of total RNA on formaldehyde containing gel
Lanes 1-3 represented RNA from transgenic cassava lines no.10, 15 and 50, respectively. Lanes 4-5 represented that from wild-type cassava cultivar KU50.

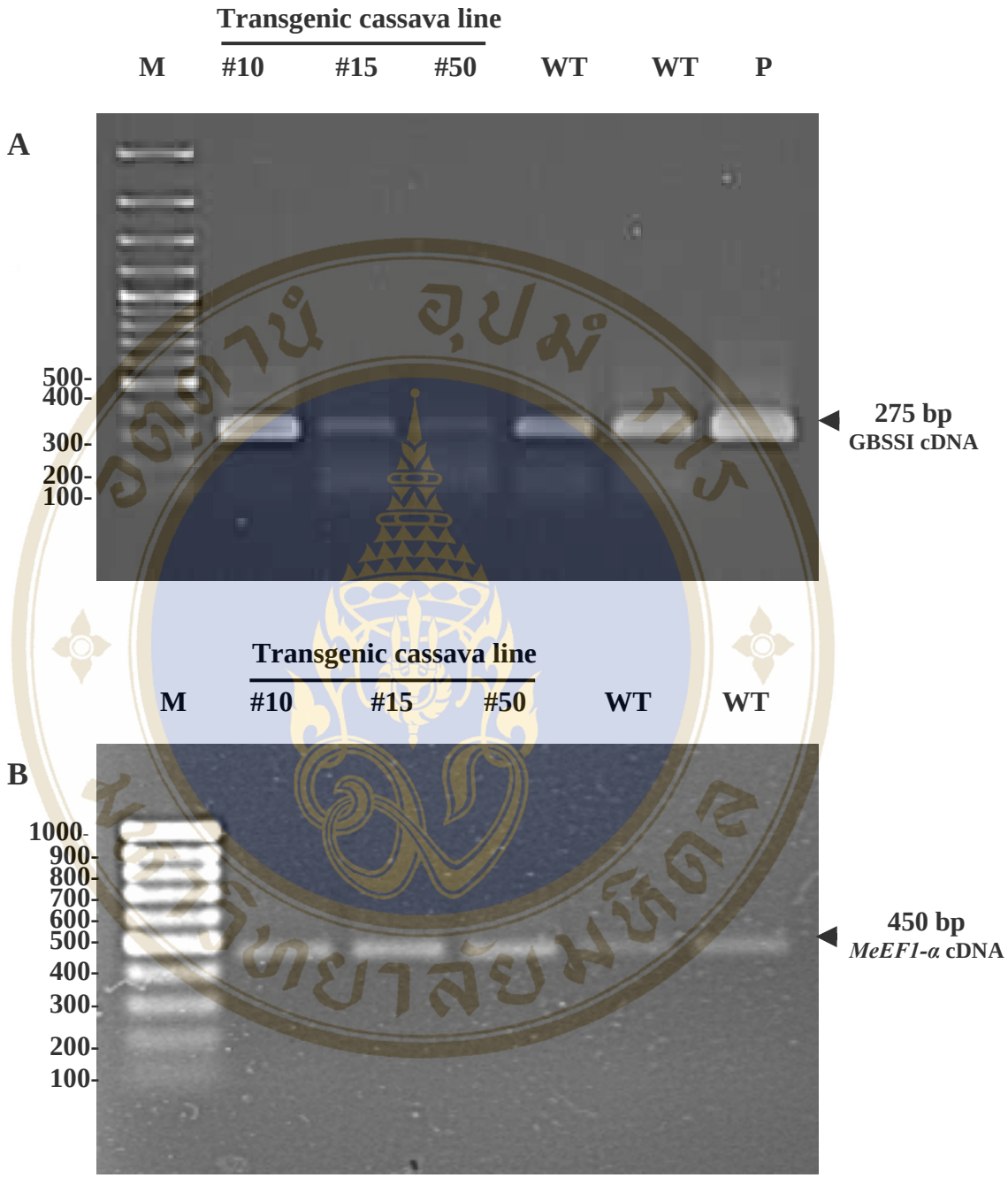


Figure 4.8 RT-PCR analysis of transgenic cassava

RT-PCR amplification of GBSSI (A) and *Me*EF1-α (B) from the cassava transgenic lines no. 10, 15 and 50 comparing with the signals obtained from wild-type cassava cultivar KU50 (WT). The sizes of molecular weight marker (M) were indicated on the left.

2.4 Iodine staining

In vitro storage roots of the transgenic line no.50 were induced by culture the uninodal segment on CSRM medium (Figure 4.9A). After 3 months, the storage root-like structure could be obtained. To investigate whether the *in vitro* storage root-like structure from the cassava transgenic line no.50 could still stored starch, iodine staining was performed (Figure 4.9B). By comparing with wild type starch granules, the granules from transgenic line appeared to be slightly brownish (Figure 4.9C and D). This should indicate the reduction on amylose content in the transgenic starch granules. Results obtained from PCR, RT-PCR, Southern blot and iodine staining confirmed that, as compared with other transgenic lines obtained from this study, the transgenic lines no.50 should represent a strong silencing clone of GBSSI gene.

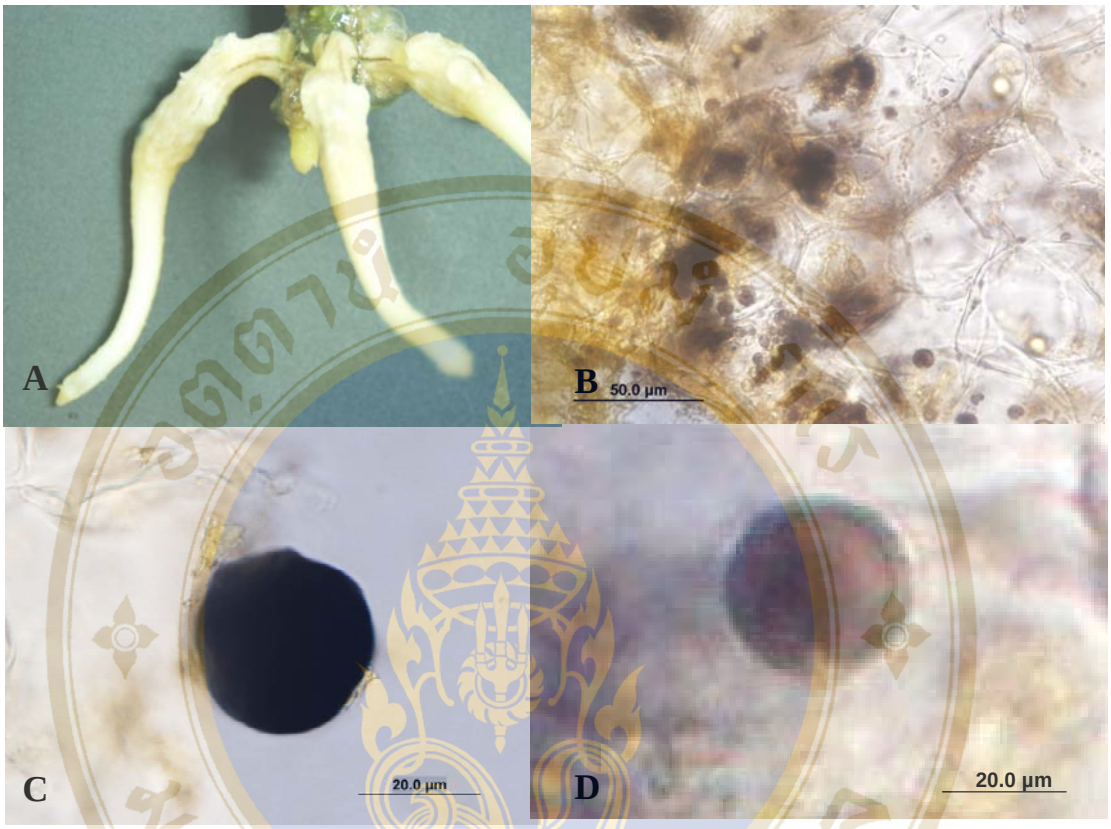


Figure 4.9 Morphological analysis and iodine staining of *in vitro* storage roots from transgenic cassava

(A) Storage root-like structure; (B) iodine staining of the cross section of storage root-like structure; (C and D) Iodine staining of starch granule from wild-type and transgenic cassava line no.50, respectively.

PART III Construction of pCAMBIA1301::p54::GBSSi::*gus*

To study gene expression ability of the phloem-specific promoter p54 and to investigate the effect of GBSSI gene suppression in other model plants, such as potato and tobacco, the plasmid vector pCAMBIA1301::p54::GBSSi::*gus* needed to be constructed. To do that, a fragment containing GBSSI inverted repeats was cut from pKANNIBAL::35S::GBSSi (Figure 3.5) using *Xho*I and *Bam*HI. Adapters containing *Nco*I and *Bgl*II sites were then ligated to the ends exposing 5'-*Xho*I and 3'-*Bam*HI sites, respectively. The blunt end product was ligated to pCR®-Blunt vector and transformed into *E.coli* strain TOP10 for multiplying propose. Confirmation for the presence of GBSSi insert in pCR®-Blunt vector was performed by *Eco*RI digestion, which yielded 2 fragments; a 3.5-kb fragment of pCR®-Blunt vector and a 1.3-kb fragment of GBSSI inverted repeat with *Nco*I and *Bgl*II adapters attached to each ends (Figure 4.10). After that, the recombinant plasmid was digested with *Nco*I and *Bgl*ll for subcloning into pCAMBIA1301::p54::*gus*. Unfortunately, the GBSSi fragment contained multiple *Bgl*II sites located in the intron region derived originally from pKANNIBAL (Figure 4.11). To solve this problem, partial digestion with *Bgl*II was carried out by varying the incubation time and digested products observed at different time were investigated by gel electrophoresis (Figure 4.12). The expected band of 1.3 kb was observed when digestion was performed for 5 to 30 min. The *Nco*I/*Bgl*II-digested fragment was gel purified, cloned into pCAMBIA1301::p54::*gus*, and transformed into *E.coli* strain DH5α. The clones carrying recombinant plasmid pCAMBIA1301::p54::GBSSi::*gus* were identified by digestion with *Nco*I and *Bam*HI, which was expected to yield 10-kb fragment of core plasmid, 1-kb fragment of p54 promoter and 1.3 kb fragment of GBSSI inverted repeats (Figure 4.13). These digested products were observed in the recombinant clone no.1, 6 and 7. The recombinant plasmid pCAMBIA1301::p54::GBSSi::*gus* would be used in biolistic-mediated transformation of tobacco as described in Part V, Topic 2.

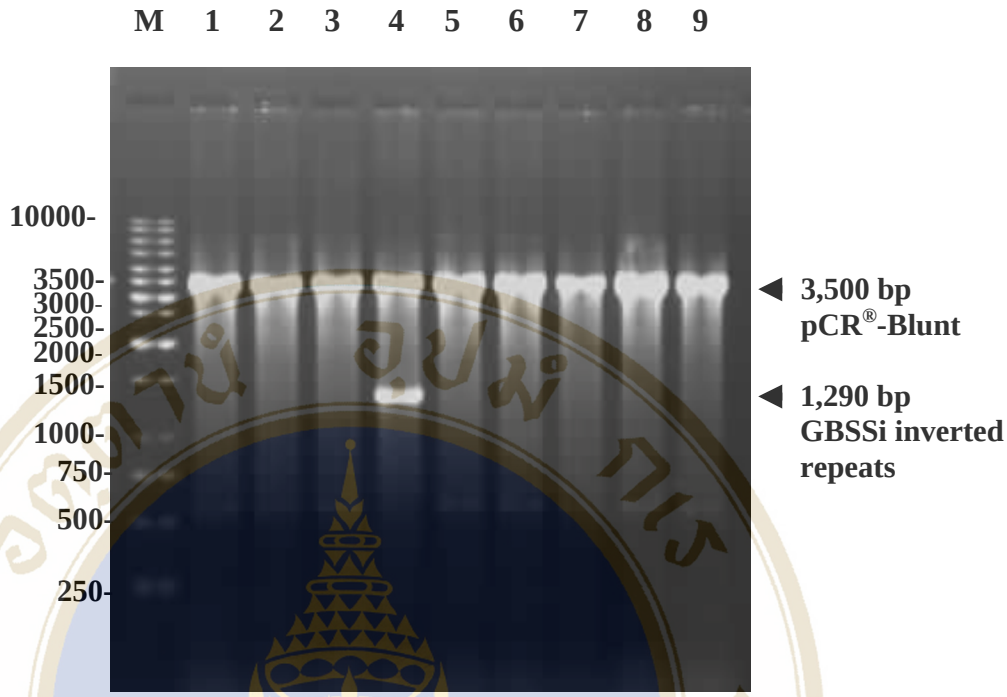


Figure 4.10 *Eco*RI digestion of recombinant pCR®-Blunt::GBSSi

Lanes1-9 represented various clones being analyzed in this study. The size of each marker band (M) was indicated on the left.

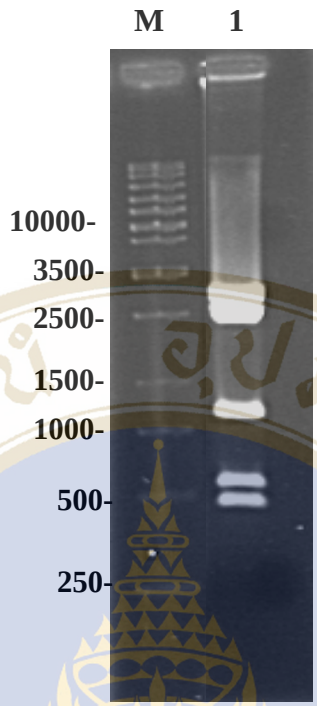


Figure 4.11 Double digestion of pCR®-Blunt::GBSSi with *Nco*I and *Bgl*II

Lane 1 represented the recombinant pCR®-Blunt::GBSSi clone no.4 from the Figure 4.10. The size of each marker band (M) was indicated on the left.

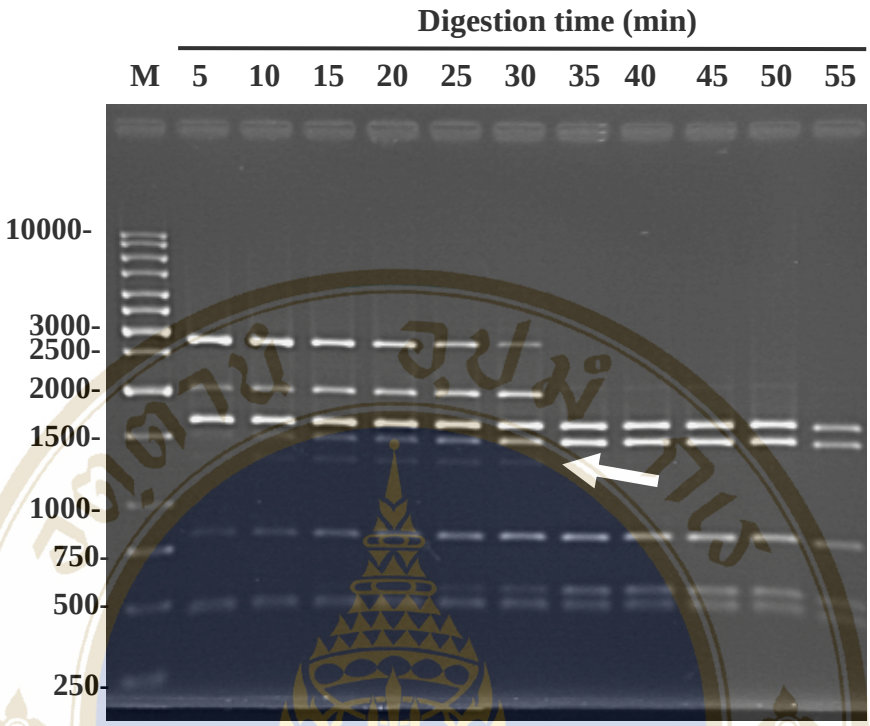


Figure 4.12 Partial digestion of pCR®-Blunt::GBSSi with *Bgl*II

The plasmid pCR®-Blunt::GBSSi was first completely digested with *Nco*I, and then subjected to partial digestion with *Bgl*II by varing the incubation time from 5 to 55 min. The expected band of 1.3 kb was indicated with arrow. The sizes of molecular marker (M, 1-kb DNA ladder (Fermentas)) were indicated on the left.

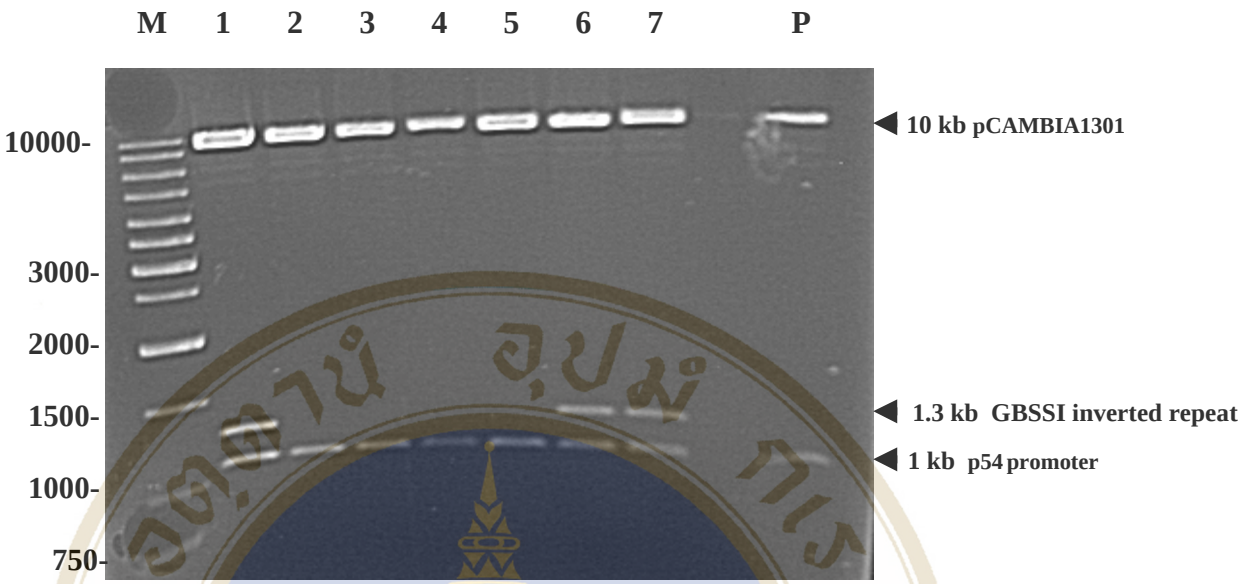


Figure 4.13 Double digestion of pCAMBIA1301::p54::GBSSi::*gus* with *Nco*I and *Bam*HI. Lanes 1-7 represented various clones being analyzed in this study in comparison with result obtained from pCAMBIA1301::p54::*gus* (Lane P). The sizes of molecular marker (M, 1-kb DNA ladder (Fermentas)) were indicated on the left.

PART IV Transformation of potato with a tissue-specific promoter from cassava

To study the effect of phloem-specific promoter, namely p54, in driving expression of foreign genes in potato, expression of GUS was monitored after transformation of potato with pCAMBIA::p54::*gus* (Figure 3.2). In addition, potato was also used as model to study the suppression effect of GBSSI gene by RNAi. Unlike in cassava, the construct used in potato was pART27::35S::GBSSi. Since the RNAi construct used in potato transformation was obtained using GBSSI sequence from cassava, additional advantage in conducting this experiment was to be able to determine whether the cassava GBSSi construct could be used to suppress endogenous GBSSI gene in potato. The first step before potato transformation experiments could be conducted, various parameters still needed to be optimized. These included the induction of *in vitro* mini-tubers and determination of the effect of hygromycin B on the shoot regeneration frequency.

1. Axenic culture of potato plants and *in vitro* mini-tuber formation

This study utilized *Solanum tuberosum* cultivar Patrone, whose axenic cultures were kindly provided by Dr. Salak Phansiri (Scientific Equipment Center, KURDI, Kasetsart University). To induce for *in vitro* mini-tubers, stem segments containing auxiliary buds, obtained from node cutting culture on PBM medium, were placed on PTM , potato mini-tuber medium (MS salt with vitamins, 5 mg/l BAP, 8% sucrose, 0.3% gelrite; pH 5.8). After 1 week in continuous light condition at 26°C, the culture was kept in the dark for 2 weeks. At this step, a small white mini-tuber could be observed. Additional incubation for 4-6 weeks in the light condition could induce further enlargement of the mini-tuber. The mini-tubers developed at the base of the shoots would turn green if being continuously incubated under the light (Figure 4.14). Iodine staining of a cross section of the *in vitro* mini-tuber indicated the presence of starch granules (Figure 4.14). This therefore indicated that the *in vitro* mini-tubers still have ability to synthesize and store starch. Availability of this system would be advantages for functional study of genes in starch biosynthesis pathway.

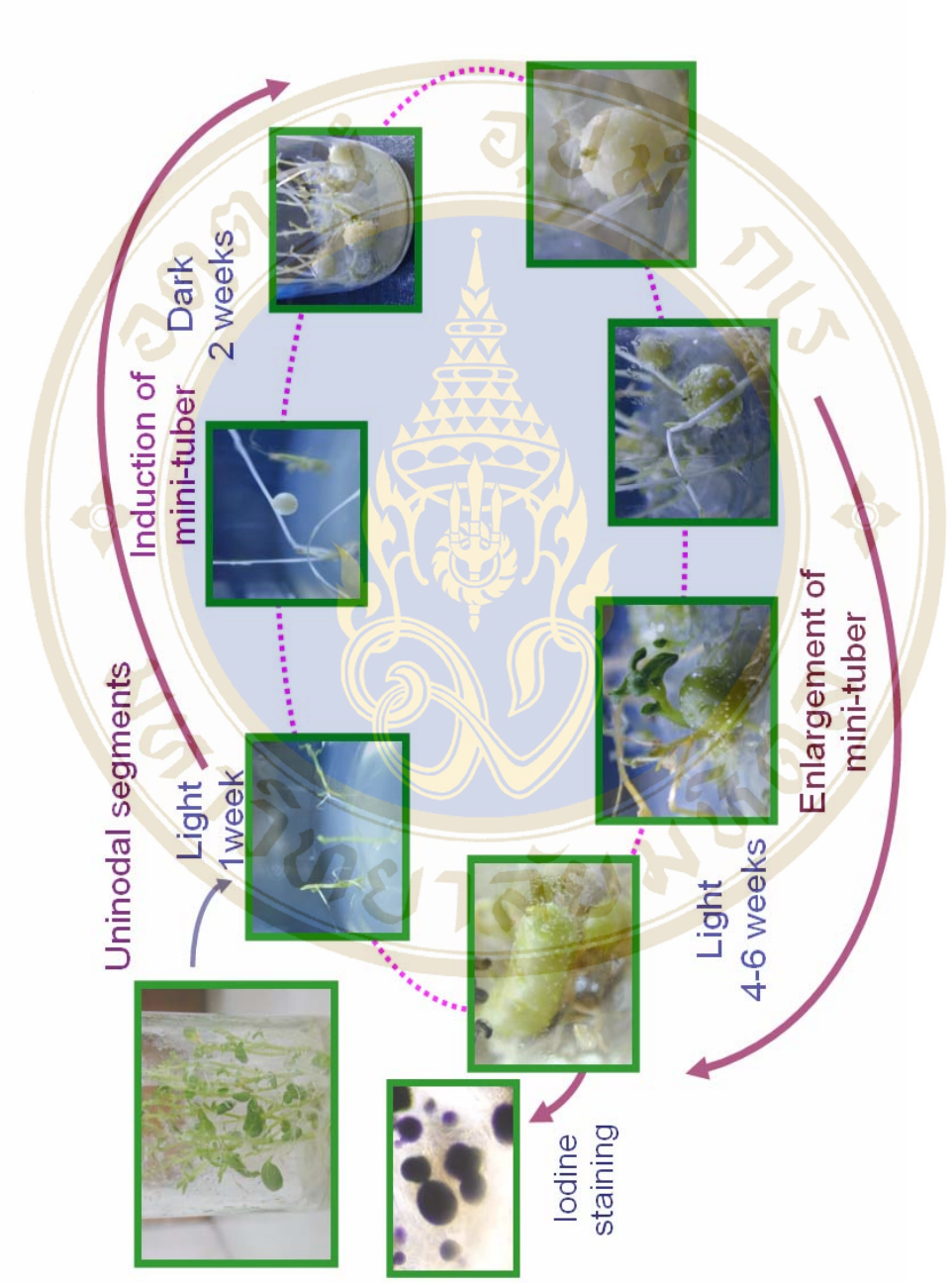


Figure 4.14 Schematic representations of steps for *in vitro* mini-tubers formation in potato.

2. Investigation of the effect of hygromycin B on potato shoot regeneration

Stem segments of potato were cultured on PBM supplemented with various concentrations of hygromycin B (5, 10 and 15 mg/l). After the culture was kept in the light at 26°C for 4 weeks, viability of the emerging shoots was investigated. The results showed that hygromycin B at the concentration of 15 mg/l could completely inhibit the shoot regeneration of potato (Figure 4.15). Therefore, selection of transgenic potato lines would be performed on PBM supplemented with 10 mg/l hygromycin B.

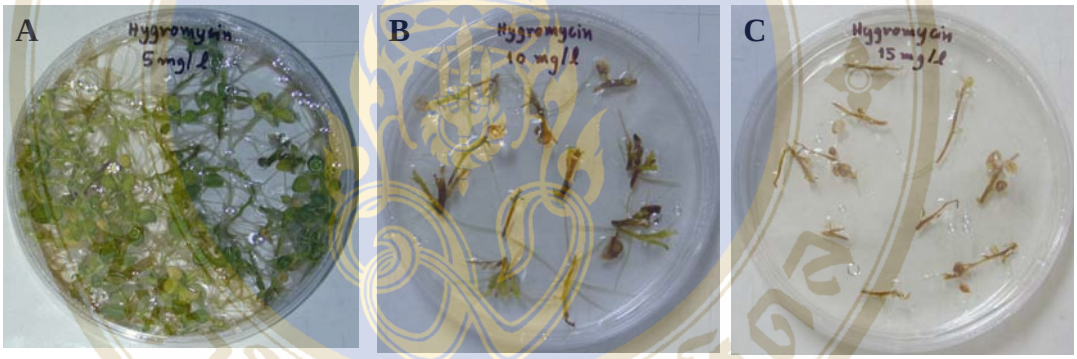


Figure 4.15 Effect of hygromycin B on potato shoot regeneration
Potato stem segments were cultured on PBM supplemented with hygromycin B at 5, 10 and 15 mg/l (A-C, respectively).

3. Functional analysis of a cassava-derived phloem-specific promoter in transgenic potato

In this study, an action of the phloem-specific promoter p54 in driving expression of foreign genes was investigated using potato as model. The reason why potato was chosen was mainly due to the availability of efficient transformation system. In addition, if the tissue-specific expression manner of p54 in transgenic potato could be observed, targeted gene expression experiments could be conducted. As a result, availability of this system in potato should benefit further characterization of genes in various biochemical pathways. In this experiment, uninodal segments of potato were transformed using *Agrobacterium* strain LBA4404 harboring pCAMBIA1301::p54::*gus*. After 2 weeks of transformation, the shoots observed on selective medium (PBM supplemented with 10 mg/l hygromycin B), were induced for regeneration of potato plants. The node-cutting segments obtained from the transgenic plants were transferred to PTM medium for mini-tuber induction. The leaves were used as source for genomic DNA and, subsequently, PCR was performed to confirm for the presence of transgene.

3.1 PCR analysis

To verify the presence of transgenes, the presence of various regions on pCAMBIA1301::p54::*gus* was determined using PCR. These included 198-bp fragment of CaMV 35S promoter, which drives expression of hygromycin resistance gene (Figure 4.16), and 1,700-bp fragment of *gus* gene (Figure 4.17). The results from PCR analysis indicated the presence of transgenes in 3 transgenic lines designated as no.17, 24 and 34. These transgenic lines were then subjected to further analysis.

3.2 RT-PCR analysis

RT-PCR was performed to determine whether the transgenes could be expressed *in vivo*. Expression of hygromycin resistance gene and *gus* gene was determined using Hygro-F & Hygro-R and G1 & G1 primers, respectively. While 600-bp PCR fragment

of *hpt* cDNA could be detected (Figure 4.18A), none of the transgenic lines was shown to carry *gus* transcript (Figure 4.18B). Since hygromycin resistance gene and *gus* gene were driven by different promoters, the lack of *gus* transcript in the potato transgenic lines could possibly result from the non-functioning of p54 promoter in potato plant.

3.3 Iodine and GUS staining

The potato transgenic lines (no.17, 24 and 34) that harbored both hygromycin resistance gene and *gus* gene did not show GUS activity in any tissues including leaf, stem and root (Figure 4.19A). In order to investigate the effect of transformation on mini-tuber formation and starch biosynthesis in this system, iodine staining was performed. As seen in figure 4.19 C & D, starch biosynthesis in these transgenic lines was not interfered because starch granules were stained blue with iodine and showed normal shape and size.

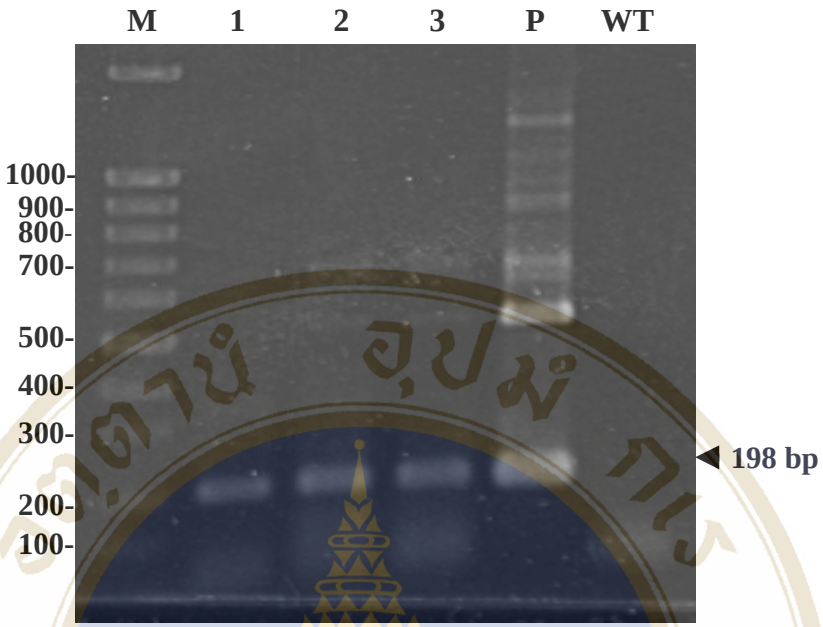


Figure 4.16 PCR amplification of CaMV 35S promoter in transgenic potato transformed with pCAMBIA1301::p54::*gus*

Lanes 1-3, the transgenic lines no.17, 24 and 34, respectively; P, plasmid pCAMBIA1301::p54::*gus*; WT, wild-type potato cultivar Patrone; M, 100-bp DNA ladder (Fermentas).

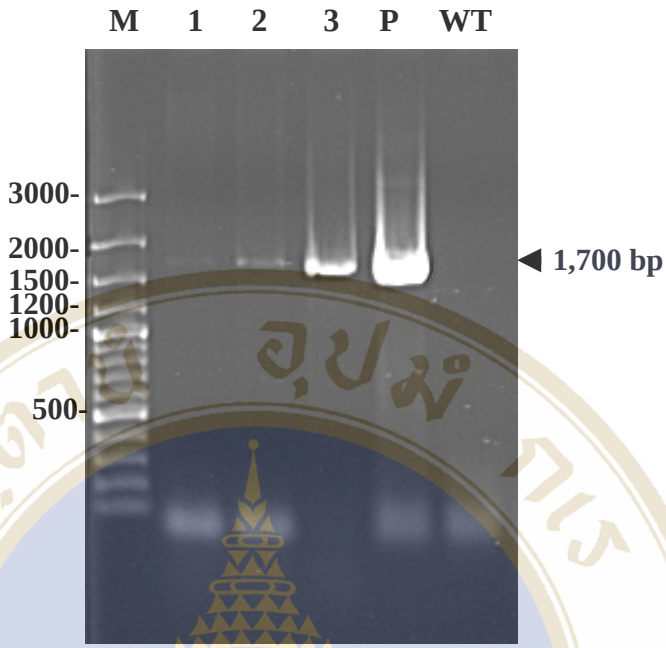


Figure 4.17 PCR amplification of *gus* in transgenic potato transformed with pCAMBIA1301::p54::*gus*

Lanes 1-3, the transgenic lines no.17, 24 and 34, respectively; P, plasmid pCAMBIA1301::p54::*gus*; WT, wild-type potato cultivar Patrone; M, 100-bp DNA ladder (Fermentas).

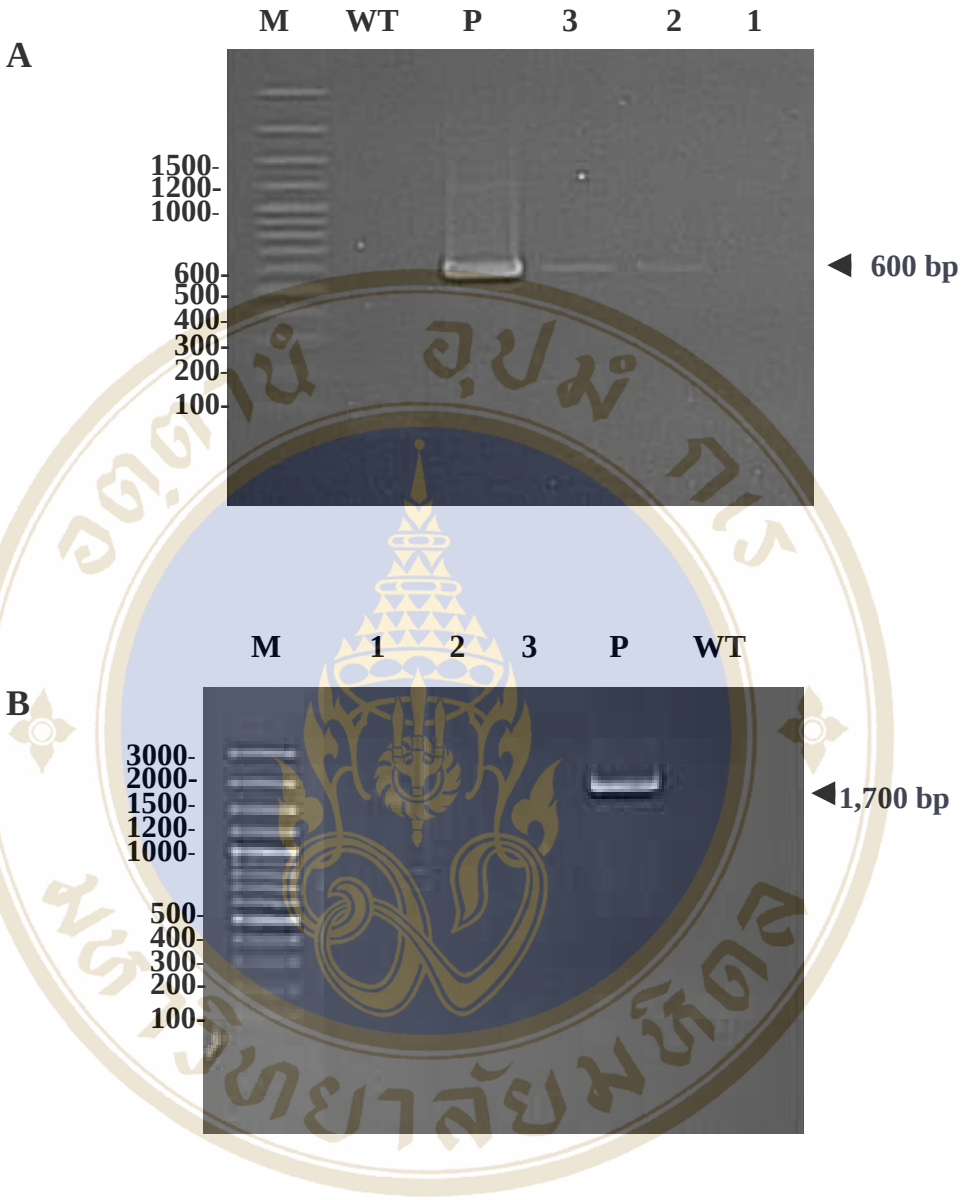


Figure 4.18 RT-PCR amplification of hygromycin resistance gene (A) and *gus* gene (B) in transgenic potato transformed with pCAMBIA1301::p54::*gus*

Lanes 1-3, the transgenic lines no.17, 24 and 34, respectively; WT, wild-type potato cultivar Patrone; M, 100-bp DNA Ladder Plus (Fermentas).

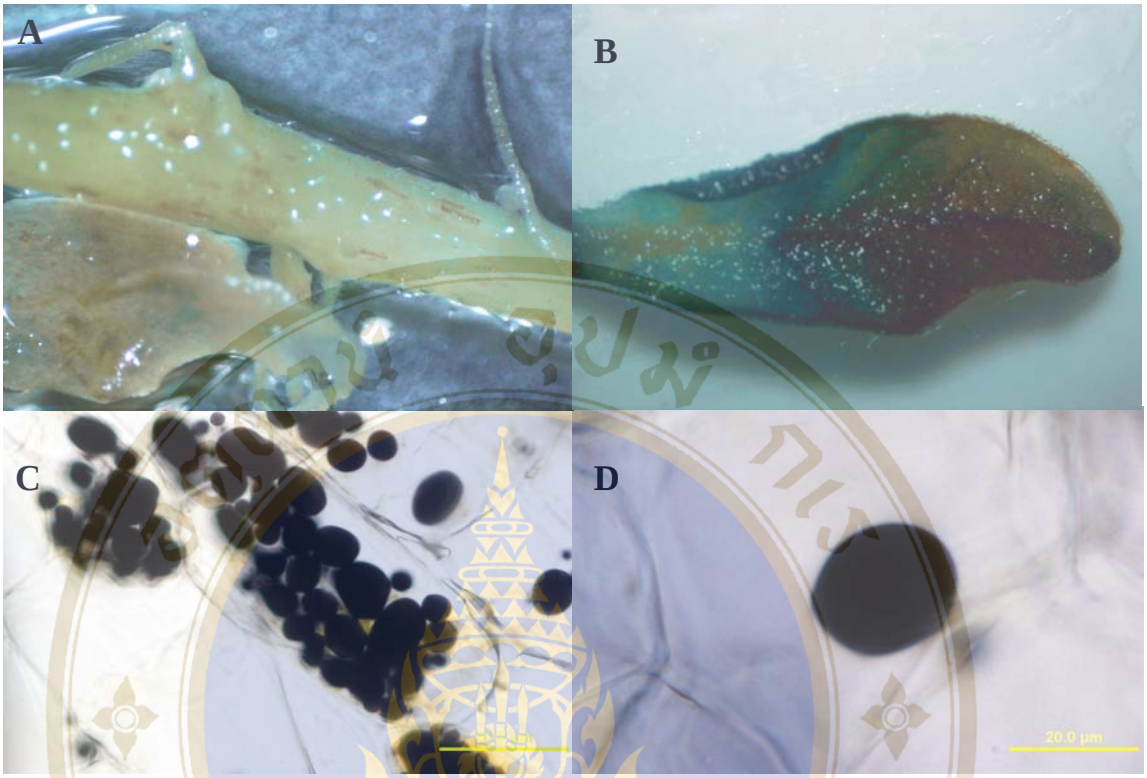


Figure 4.19 GUS and iodine staining of transgenic potato transformed with pCAMBIA1301p54::*gus*

GUS staining of (A) stem of potato transgenic lines and (B) leaf of transgenic tobacco expressing *gus* gene; (C and D) Iodine staining of mini-tubers from transgenic potato.

4. *Agrobacterium*-mediated transformation of potato with a cassava GBSSI-RNAi construct

In this study, the vector pART27::35S::GBSSi was transformed into potato using *Agrobacterium*-mediated transformation. The plasmid pART27::35S::GBSSi was constructed from partial fragment of a cassava GBSSI gene. However, the function of this plasmid in endogenous GBSSI gene suppression via RNAi was studied in potato.

4.1 PCR analysis

After *Agrobacterium*-mediated transformation, integration of CaMV 35S promoter into genome of transgenic potato was monitored by PCR. An amplified fragment of 198 bp could be observed in the transgenic lines no.3 and 16 (Figure 4.20). The frequency of transformation was found to be 12.5% of total putative transgenic lines.

4.2 Iodine staining

The putative transgenic lines were subcultured and induced for *in vitro* mini-tubers function. Size and shape of the mini-tubers from transgenic lines were the same as those from wild-type plants (data not shown). A cross section of the mini-tuber was stained with iodine. Interestingly, the iodine-stained starch granules from both wild type and transgenic lines appeared dark blue with iodine (Figure 4.21). Thus, it would be possible that amylose content in the transgenic potato was not significantly altered. This evidence might result from an inefficient suppression of GBSSI gene by RNAi. Since the vector pART27::35S::GBSSi was constructed using sequence from cassava, the result might be suggest that partial fragment of a cassava GBSSI gene could not effective enough to suppress endogenous GBSSI gene in potato.

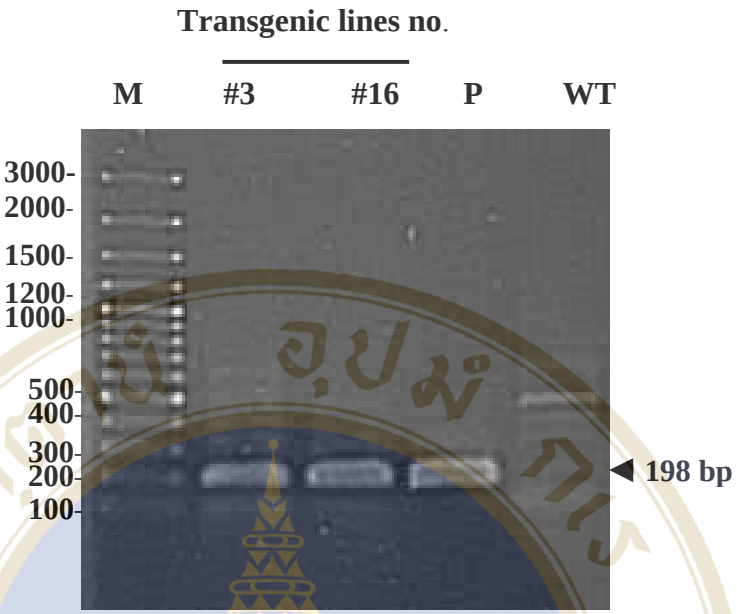


Figure 4.20 PCR amplification of CaMV 35S promoter in transgenic potato transformed with pART27::35S::GBSSi

PCR was performed using CaMV35S-F & CaMV35S–R primers. P, plasmid pART27::35S::GBSSi; WT, wild-type potato cultivar Patrone; M, 100-bp DNA Ladder Plus (Fermentas).

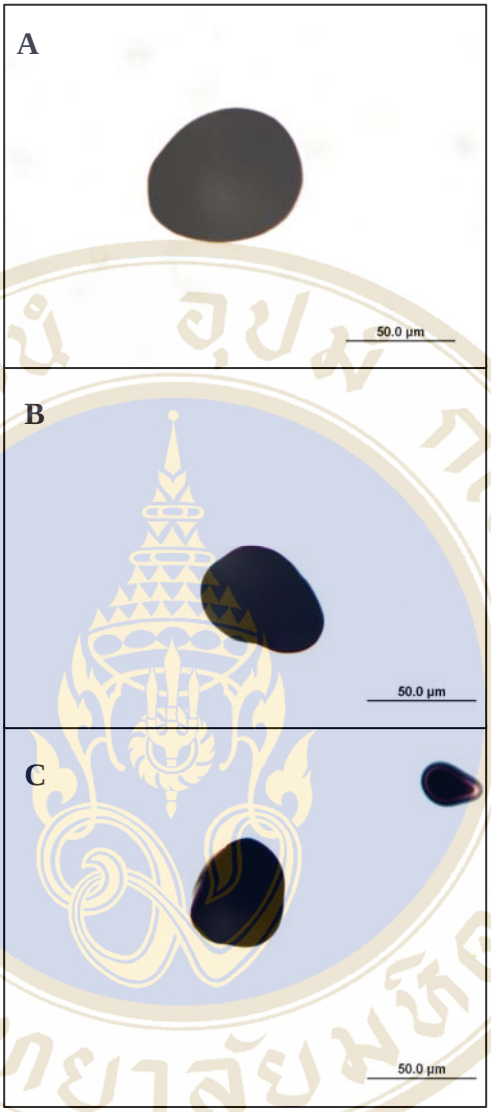


Figure 4.21 Iodine staining of starch granules from transgenic potato transformed with pART27::35S::GBSSi

(A) Starch granule from wild type potato cultivar Patrone; (B and C) Starch granules from transgenic line no.3 and 16, respectively.

PART V Expression analysis of cassava genes in tobacco

1. Functional analysis of cassava-derived phloem-specific promoter in transgenic tobacco

Tobacco is commonly used as plant model to study function of genes. In this study, the gene expression ability of the phloem-specific promoter p54 was studied using tobacco as the model. In addition, tobacco was also transformed with a construct containing p54 promoter and GBSSI-RNAi fragment.

In this experiment, tobacco was transformed by particle bombardment using leaf discs obtained from 1 to 2 month-old plants. Before bombardment, the cut leaves were placed, with their abaxial side facing upward, on RMOP medium and then pre-plasmolysed overnight. Bombardment was carried out using the following parameters; 28 in. of mercury pressure, 1,350 psi. pulse, and 9 cm target distance. After bombardment, the leaves were post-plasmolysed overnight, cut into small pieces (1x1 cm), and then transferred to RMOP medium. Small shoots observed after 3-4 weeks were then transferred to RM medium for regeneration. Within 1-2 months, healthy tobacco plants could be obtained and leaves from these putative lines were subjected to DNA extraction and PCR analysis.

1.1 PCR analysis

After the plasmid pCAMBIA1301::p54::*gus* was bombarded into tobacco leaf discs, PCR was performed to confirm the integration of transgene in tobacco genome. By using CaMV35S-F and CaMV35S-R primers, amplification of 198-bp fragment of CaMV 35S promoter was observed in various transgenic lines of tobacco (Figure 4.22A). The lines that contained CaMV 35S promoter were subjected to PCR amplification of *gus* gene. The result showed that only 24% of the transgenic lines that contained CaMV 35S promoter in their genome gave positive result with *gus* primer (Figure 4.22B).

1.2 RT-PCR analysis

RT-PCR was performed to confirm that genes in the expression cassette could be appropriately expressed. The results from RT-PCR analysis showed that the tobacco transgenic lines expressed hygromycin resistance gene, while expression of *gus* gene could not be detected (Figure 4.23). It should be noted that the *gus* gene in pCAMBIA1301::p54::*gus* was driven by a phloem-specific promoter (p54), while hygromycin resistance gene was driven by CaMV 35S promoter. Therefore, the absence of *gus* transcript in the tobacco transgenic lines may result from the nonfunctioning of the p54 promoter in tobacco. This was confirmed by subjecting the leaves and stems of tobacco transgenic lines to histochemical assay. According to Figure 4.24, none of the lines was shown to possess GUS activity.

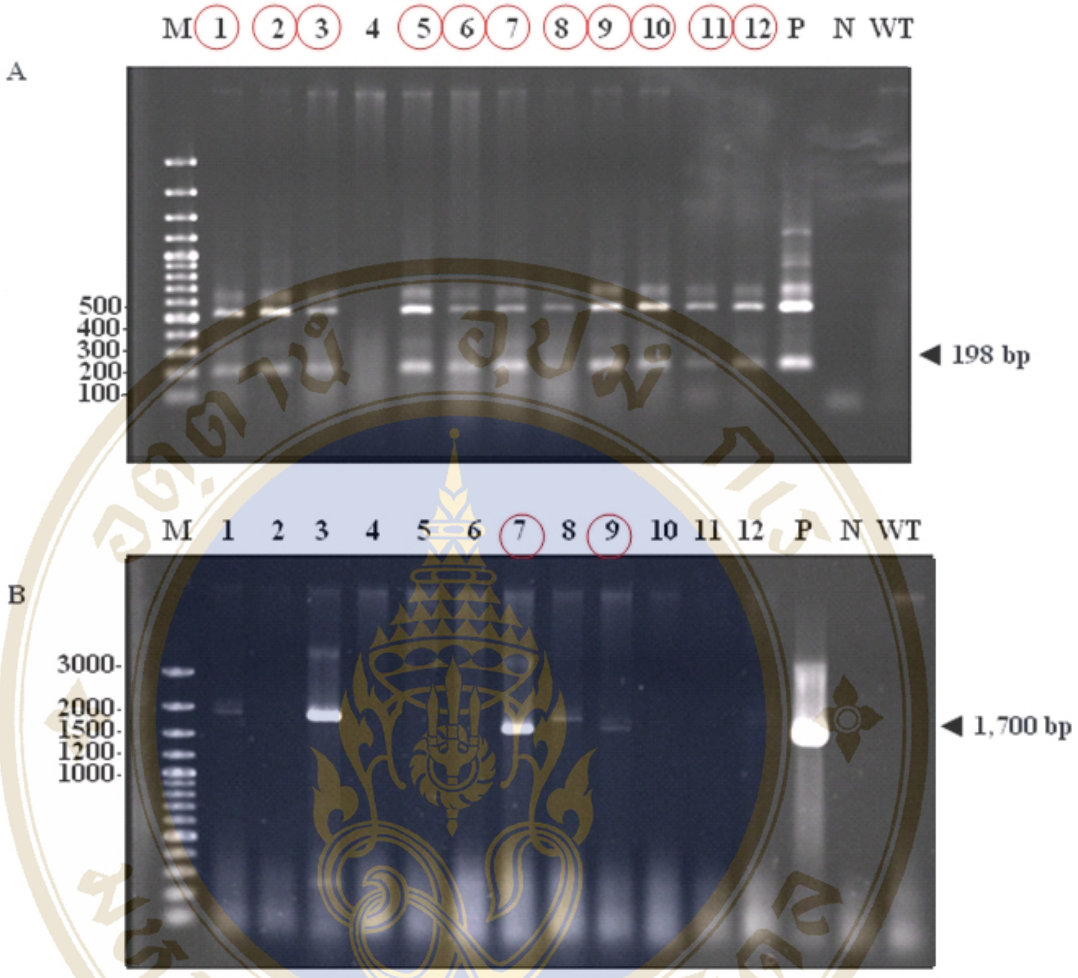


Figure 4.22 PCR analysis of transgenic tobacco bombarded with pCAMBIA1301::p54::*gus*

PCR was performed using primers specific to a region in CaMV 35S promoter (A) and *gus* gene (B). Lanes 1-12 represented particular lines of transgenic tobacco. P, pCAMBIA1301::p54::*gus*; WT, wild-type tobacco cultivar Ottawa; M, 100-bp DNA Ladder Plus (Fermentas). Red circle indicated the line with expected PCR product.

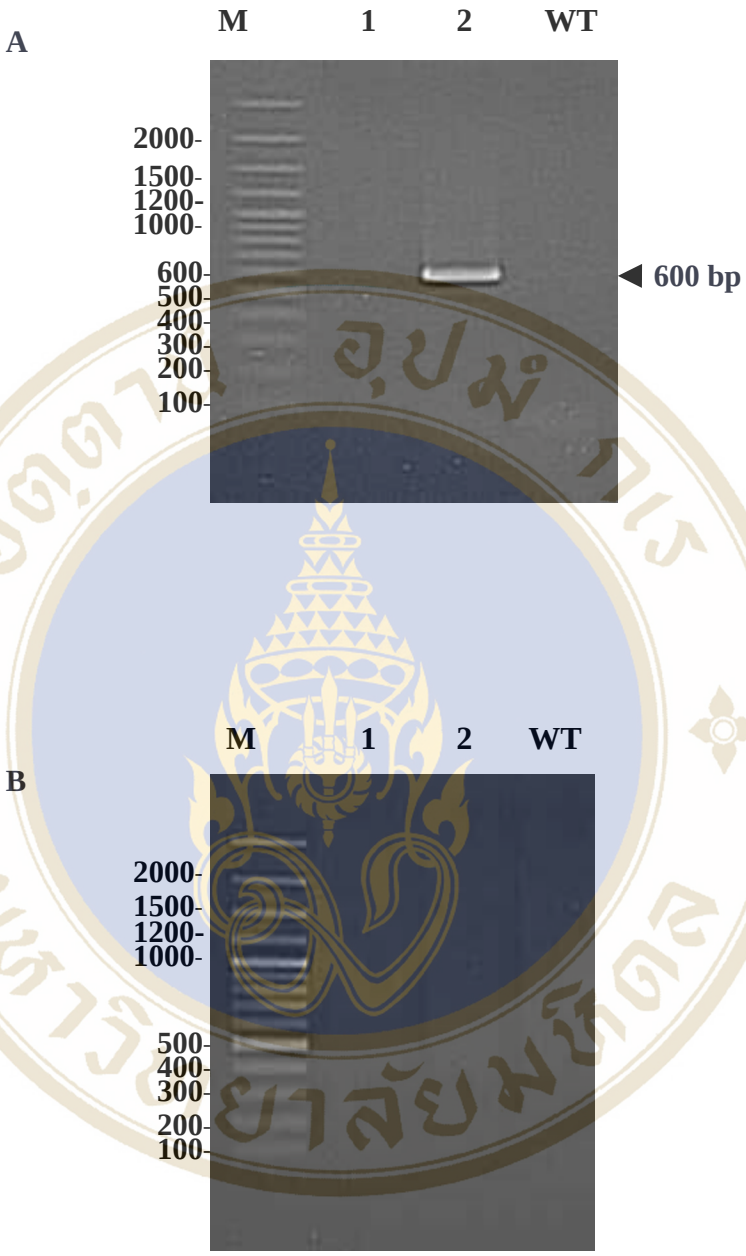


Figure 4.23 RT-PCR analysis of hygromycin resistance gene (A) and *gus* gene (B) in transgenic tobacco transformed with pCAMBIA1301::p54::*gus*

Lanes 1 and 2 represented the transgenic lines no.7 and 9, respectively. WT, wild-type tobacco cultivar Ottawa; M, 100-bp DNA Ladder Plus (Fermentas).

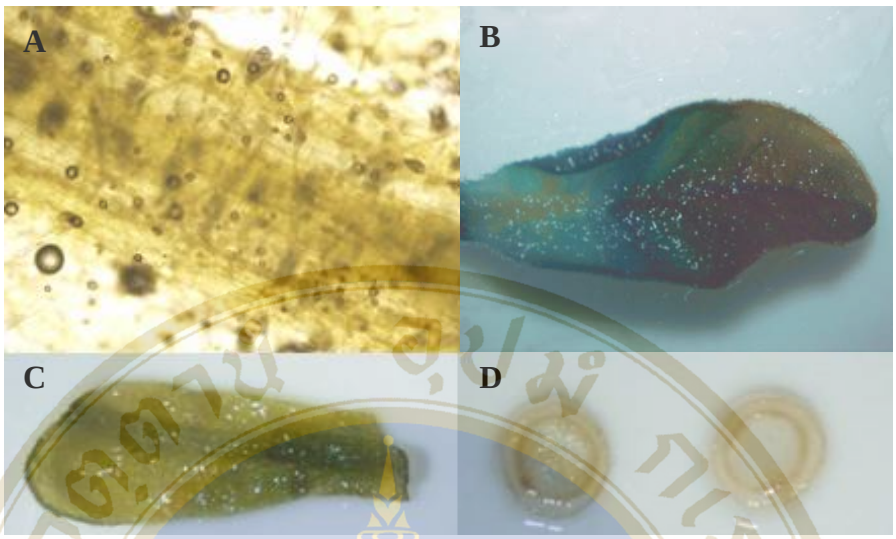


Figure 4.24 GUS staining of transgenic tobacco

(A) Mid-rib, (C) leaf, (D) stem cross sections of a transgenic tobacco; (B) transgenic tobacco expressing *gus* gene.

2. Investigation of heterologous expression of GBSSI-RNAi fragment driven by a cassava-derived phloem-specific promoter

The objective of this experiment was to determine whether the phloem-specific promoter p54 could be used to drive expression of GBSSI-RNAi fragment, thus resulting in the suppression of tobacco GBSSI gene. The vector pCAMBIA1301::p54::GBSSi::*gus* was transformed into tobacco via particle bombardment. The shoots observed on selective medium (RMOP supplemented with 20 mg/l hygromycin B) were dissected and transferred to RM medium for regeneration.

2.1 PCR analysis

The leaves of putative lines were subjected to DNA extraction and PCR analysis. To verify the presence of transgene, PCR was performed using GBSSiF1+GBSSiF2 primers, which were expected to yield a 1,290-bp fragment. The plasmid pCAMBIA1301::p54::GBSSi::*gus* and pKANNIBAL::GBSSi were used as positive control. By screening all the putative lines with PCR, only the line no.3 showed an amplified product, whose size was closely similar to that observed in positive lanes (Figure 4.25). As the size of amplified product in the line no.3 was smaller than the expected size of 1,290 bp, this line was not the expected transgenic lines. However the line no.3 was still used as candidate for further analysis including GUS and iodine staining.

2.2 Iodine and GUS staining

GUS staining was performed using stems and leaves from 2-month old tobacco plant. As expected the GUS was not observed in stems and leaves of this tobacco line no.3 (Figure 4.26). To observe starch accumulation, iodine staining was performed using stems of 2-month old tobacco. The result showed that starch granules were

stained blue, thus indicating that composition of starch in this tobacco line no.3 was not affected.

Since the transgenic tobacco line was not obtained in this experiment, the inability of cassava GBSSi fragment in tobacco GBSSI gene suppression was not conclude.

Figure 4.25 PCR analysis of transgenic tobacco bombarded with pCAMBIA1301::p54::GBSSi::*gus*

PCR was performed using primers specific to a region in GBSSI-RNAi cassette**.** Lanes 1-7 represented particular lines of transgenic tobacco. P1, pCAMBIA1301::p54::GBSSi::*gus*; P2, pKANNIBAL::35S::GBSSi; WT, wild-type tobacco cultivar Ottawa; M, 100-bp DNA Ladder Plus (Fermentas).

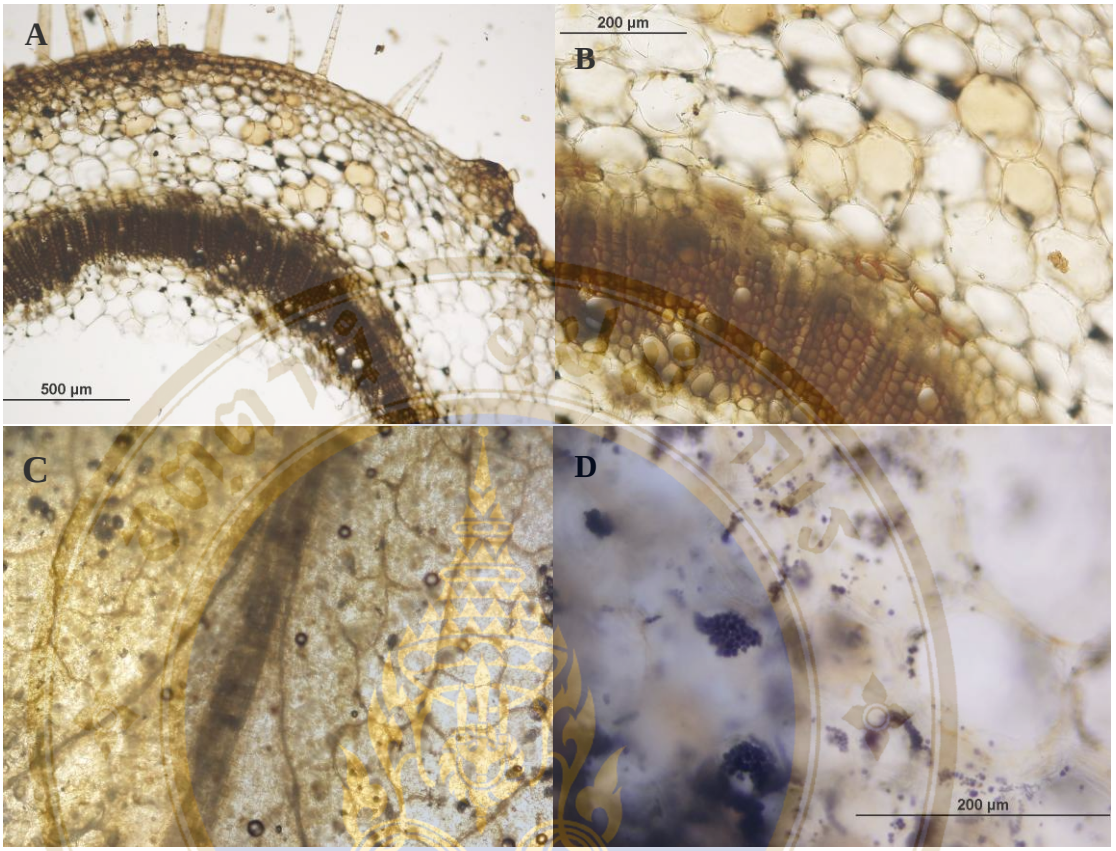


Figure 4.26 GUS and iodine staining of transgenic tobacco bombarded with pCAMBIA1301::p54::GBSSi::*gus*

GUS staining of stem (A and B) and leaf (C) leaf and iodine staining (D) of a transgenic tobacco lines.

CHAPTER V
DISCUSSION

PART I Establishment of a cassava tissue culture system for gene function study

Cefotaxime was suitable for elimination of Agrobacterium while retaining acceptable induction efficiency of cassava shoot organogenesis

In this study, two objectives were established when studying the effect of antibiotics. The first goal was to identify an antibiotic that would be most effective against *Agrobacterium* and the second goal was to determine whether that particular antibiotic would affect shoot organogenesis in cassava. In addition to kanamycin and hygromycin B, the effects of cefotaxime, carbenicillin and augmentin on *Agrobacterium* growth suppression and cassava shoot organogenesis were monitored. For the first objective, the effect of these antibiotics on the growth of various *Agrobacterium* strains including EHA105, AGL1 harboring pCAMBIA1301 and LBA4404 harboring pCAMBIA1301::p54::*gus* were monitored. The results showed that cefotaxime at 300 mg/l would be most effective against these *Agrobacterium* strains. Similar to the evidence observed in *Nicotiana tabacum*, cefotaxime was also reported as the most effective agent at suppressing the growth of *Agrobacterium* strain LBA4404 (Shackelford & Chlan, 1996). For the effect of antibiotics on the shoot organogenesis, it was found that with increasing amount of antibiotics the shoot organogenesis of both cassava cultivars would be greatly affected. However, the effect would be greatly pronounced in KU50 cultivar. When compared with carbenicillin and augmentin, cefotaxime was found to be more toxic to the shoot organogenesis of cassava. However, based on the fact that cefotaxime is cheaper and more effective against various *Agrobacterium* strains, it would be used in subsequent experiments involving *Agrobacterium*-mediated transformation of cassava.

For kanamycin and hygromycin B, at the concentration of 40-100 mg/l the shoot organogenesis of KU50 and Hanatee would be completely inhibited. According to Li *et al.* (1996), adventitious shoot of some cassava genotypes could still be inhibited in the presence of hygromycin B at concentration as low as 5 mg/l. In this study, however, acceptable induction efficiency of shoot organogenesis could be obtained when using kanamycin at 15-25 mg/l and hygromycin B at 10-20 mg/l. Therefore, in combination with cefotaxime at 300 mg/l, these concentrations of kanamycin and hygromycin B would be used in subsequent experiments involving *Agrobacterium*-mediated transformation of cassava. Although there was a report indicating that hygromycin B was inefficient to be used as selectable marker for cassava embryogenic cell (Christian *et al.*, 1996), the utilization of hygromycin B in this study was essential especially when selecting for transgenic lines harboring pCAMBIA1301.

Based on the negative effect of antibiotics, particularly kanamycin and hygromycin B, on the cassava shoot regeneration, alternatively, the addition of antibiotics could be omitted and selection of putative transgenic lines could be performed using PCR (Vetten *et al.*, 2000). With the added advantages of PCR, this procedure was used throughout this study.

In vitro storage root formation of the cassava cultivar KU50 was developed to serve as a tool in gene function study

Numerous reports on cultivation of cassava have been published, but only small amount of information on the *in vitro* storage root formation is available. In comparison with storage roots harvested from field-grown cassava plants, the *in vitro* storage roots not only showed exo-morphological similarity but also possessed cortical parenchyma that functions as a storage and massive deposition site of starch granules. However, it has been mentioned that the *in vitro* storage roots could be obtained only under certain conditions (Medina *et al.*, 2007). The first report on the induction of *in vitro* storage roots of cassava was done by Medina *et al.* (2007). Their protocol was followed to determine whether the *in vitro* storage roots of cassava cultivar KU50 could be obtained.

Based on exo-morphological observation, storage roots obtained from both *in vitro* and field-grown plants were brown, furrowed and covered with scaly peridermis. In addition, starch, detected by staining with iodine, was also observed in the *in vitro* storage roots. According to anatomical analysis, parenchyma cells in the secondary growth region of *in vitro* storage roots were filled with starch granules. Results obtained from the work of Medina *et al*. (2007) further indicated that deposition of starch granules in the *in vitro* storage roots was confined to cortical tissue. These results therefore suggested that the ability to accumulate starch is also preserved in the storage roots obtained *in vitro*.

In term of production period, it took 1-3 months to obtain *in vitro* storage roots from the cassava cultivar KU50, while 29 different cultivars tested by Medina *et al*. (2007) required only 15 days. However, availability of this system in KU50 cultivar is still considered advantageous. Not only that it could be used to study starch biosynthesis in controlled environmental conditions, but public concerns regarding the unforeseen risks of transgenic cassava plants would be minimized due to the lack of having to transfer the plantlets to a greenhouse.

Somatic callus was established to serve as an alternative system for gene function study in cassava

The use of embryogenic suspensions in genetic transformation is advantageous due to availability of an efficient selection system of transgenic cells in the liquid medium. With this system, the risk in regenerating chimeric plants is also lower when compared with other techniques that involve organogenesis from organized tissues (Gonzalez *et al.*, 1998). Based on the disadvantages of cotyledon in the low regeneration capacity and the long span of cultivation *in vitro* (at least 2 months), utilization of embryonic suspension as starting material in cassava transformation would be more preferable. In this study, development of callus culture from somatic embryo cluster of the cassava cultivar KU50 was established. The somatic embryo cluster was found to be able to yield the callus culture in CCM medium. Within 2 months, the callus culture further developed to mature callus. In the liquid medium, two kinds of callus; globular-like structure and cluster callus, were observed based on

the differences in morphology and starch content. When staining with iodine, the cluster callus appeared dark blue in most of the area, while the globular callus showed a light blue color in the center. Morphological analysis of the cross section of globular and cluster callus further indicated that the globular callus should be of single origin whereas the cluster callus might result from the aggregation of immature somatic embryo. In addition to cotyledons, callus observed in the suspension culture could be used as starting material in cassava transformation.

Acetosyringone promoted the growth of A. tumefaciens strains EHA105 and AGL1

To enhance transformation efficiency via the growth of *Agrobacterium*, the effect of acetosyringone was investigated. In this experiment, an enhanced effect of acetosyringone on the growth of *Agrobacterium* strain EHA105 and AGL1 was observed. This finding was in contrast with the previous report, which indicated that the *Agrobacterium* that possess the genetic background of C58, such as EHA105 and AGL1, should have growth retardation with acetosyringone, and have low efficiency of occurrence of avirulent mutants following exposure to acetosyringone (Fortin *et al.*, 1992).

PART II Suppression of GBSSI gene in cassava by RNA interference

Prior to cassava transformation, various factors that are known to influence the efficiency of biolistic-mediated transformation were optimized. These include size of gold particle, amount of DNA, gas pressure, target distance, number of bombardment, pre- and post-plasmolysed time. Results obtained from this study indicated that the increasing number of bombardment had a negative effect on the regeneration efficiency, possibly due to lethal disruption of the cell membranes. It should be noted that in this study, the difference in target distance between 6 or 9 cm did not significantly affect the formation of shoots in transgenic cassava. The 20 hr of pre- and post-plasmolysed time gave the beneficial effect assuming from the reduction of turgor pressure that allowed the particles to penetrate the cell without causing damage (Zhang *et al.*, 2000).

The cassava transformation was performed using *Not*I digested fragment of pKANNIBAL::35S::GBSSi. The obtained fragment, namely GBSSi, then contained CaMV 35S promoter, inverted repeats of partial GBSSI gene from cassava flanking an intron from pyruvate orthophosphate dikinase gene, and an octopine synthase (*ocs*) terminator. As observed in this study, the shoot regeneration efficiency of transgenic cassava was relatively low and only three transgenic cassava lines carrying GBSSi transgene(s) could be selected. The presences of GBSSi in these transgenic lines were confirmed by PCR as well as Southern blot. Additionally, suppression of endogenous GBSSI gene was supported by RT-PCR. Among the three transgenic cassava lines, the line no. 50 was observed to have lowest level of GBSSI transcript and smallest copy number of GBSSi transgene. The observation that multiple copies of RNAi fragment do not necessarily affect endogenous gene expression at a greater level agrees with results observed in various plants (Kohli *et al.*, 1998). This could result from unstable expression of transgene due to the presence of multiple copies being integrated at the same locus or from position effects that could eventually lead to silencing of the transgene (Kohli *et al.*, 1998).

As mentioned that cassava transformation was performed using a *Not*I fragment containing essential elements for GBSSi expression rather than the whole pKANNIBAL::35S::GBSSi vector, the success of this experiment in cassava was in consistent with the work of Yao *et al.* (2006), who observed that linear transgene cassette lacking backbone sequences could be integrated into the wheat genome as effectively as could circular plasmids. These therefore proved that the lack of vector backbone sequences had no effect on the integration and inheritance of transgenes in the host genomic DNA. In addition, it was suggested that removal of the vector backbone sequence could also result in the reduction of concatemerization prior to transgene integration, thus allowing efficient integration of genes into plant genomes (Yao *et al.*, 2006). Since the *Not*I fragment transformed into cassava contained no antibiotic resistance gene, the transgenic lines obtained could therefore be considered marker-free. In term of advancement in plant research, this system could repeatedly be used to introduce multiple traits into the same line. In term of public concerns regarding the use of genetically modified plants, the risks caused by antibiotic

resistance genes would be minimized and this would therefore result in safe utilization of transgenic lines.

To confirm that suppression of GBSSI gene in the three transgenic lines of cassava would more or less affect amylose content, *in vitro* storage roots were induced and then subjected to starch determination by iodine staining method. The formation of blue-black complex between triiodide ions in the I_2/KI solution and amylose coils generally indicates the normal amylose content, while the reddish-brown color, as observed in the transgenic line no.50, indicates the low amylose content (Yu *et al.*, 1996). However, the formation of dark blue complex in the other two transgenic lines does not necessarily indicate the normal amylose content since sensitivity of the method used in this study is not high enough to detect a minute change in amylose content. Based on the convenience and suitability of this method for small amount of sample, it was chosen as the method of choice to preliminary determines whether amylose content in the cassava transgenic lines would be affected. To further confirm the results observed in this study, a quantitative analysis of amylose content will have to be performed. Alternatively, the normal amylose content in the two cassava transgenic lines could result from the action of other starch synthase isoforms (SSI, SSII and SSIII), in conjunction with the remaining GBSSI activity, in maintaining stable amylose level.

PART III Investigation of the effect of a phloem-specific promoter in potato and tobacco

The p54 promoter, derived originally from cassava, has been shown to be able to drive expression of genes in a tissue-specific expression manner. Similar to the results observed in cassava, expression of p54::*uidA* (*gus*) was also observed in phloem, cambium and xylem vessels of vascular tissues from leaves, stems, and roots of transgenic *Arabidopsis* (Zhang *et al.*, 2003a). To investigate the effect of p54 promoter in other model plants, the vector pCAMBIA1301::p54::*gus* was transformed into potato and tobacco. Selection of putative transgenic lines was performed using selective medium and also PCR analysis. Based on the results obtained from RT-PCR and histochemical analysis, *gus* expression could not be detected in putative lines of

potato and tobacco. Since expression of *gus* gene is driven by p54 promoter, the lack of *gus* transcript and GUS activity in the transgenic lines could result from the non-functioning of the p54 promoter. When using *Agrobacterium* transformation, the right border would be transferred into plant genome prior to the left border (Jen & Chilton, 1986). When consider the orientation of genes in pCAMBIA1301::p54::*gus*, hygromycin resistance gene was observed near the left border, while *gus* gene was closer to the right border. Since the transcript of hygromycin resistance gene could be detected by RT-PCR, the result therefore implied that the whole expression cassette located between the left and right borders was successfully transferred into genome of potato and tobacco transgenic lines. Therefore, the inability to detect *gus* gene expression in this experiment possibly result from the non-functioning of p54 promoter rather than the failure of *gus* gene transfer. As far as the literature concerned, there is no report on gene expression ability of p54 promoter in potato and tobacco.

In this study, the non-functioning of p54 promoter in tobacco was further confirmed by transformation with pCAMBIA1301::p54::GBSSi::*gus*. Since normal amylose content could still be observed in the transgenic tobacco line, it has been hypothesized that endogenous expression of the tobacco GBSSI gene was not affected due to the lack of GBSSi fragment and this could result mainly by the inactivity of p54 promoter in tobacco. In tobacco, suppression of GBSSI gene via RNA interference could not be studied.

PART IV Constitutive suppression of GBSSI gene via RNA interference in potato

Due to the non-functioning of the p54 promoter in potato, suppression of the potato GBSSI gene via RNA interference was studied using constitutive CaMV 35S promoter. In this experiment, *Agrobacterium*-mediated transformation of potato using the binary vector pART27::35S::GBSSi was carried out. To confirm the presence of GBSSi transgene in the potato transgenic lines, PCR amplification of various regions on the pART27::35S::GBSSi was performed. Subsequently, amylose content in the *in vitro* mini-tubers of transgenic potato was determined using iodine staining method.

Based on the results obtained from this study, two transgenic lines were shown to carry GBSSi transgene, however, their amylose content was observed to be relatively the same as in wild-type plants. Therefore, expression of endogenous GBSSI gene in these transgenic lines may not be interfered by the presence of cassava GBSSI-RNAi fragment. Based on nucleotide sequence comparison, the cassava GBSSI cDNA showed 74% identity with GBSSI from potato and 60-72% identity with GBSSI from other plants. By transforming potato with an antisense GBSSI cDNA from cassava, partial or complete inhibition of endogenous GBSSI gene in the potato transgenic lines could be observed (Salehuzzaman *et al*., 1993). The result therefore indicated that the cassava GBSSI cDNA could be used to effectively inhibit expression of endogenous GBSSI gene in potato. By subjecting starch granules from these potato antisense lines to iodine staining, it could be observed that amylose accumulation in the entire granule was not equally affected (Kuipers *et al.,* 1994). In fact, the antisense starch granules of varying degree of GBSSI expression were shown to contain a blue-staining core of varying size, while the outer area appeared red and/or brownish in the presence of iodine. This finding indicated that reduced GBSS gene expression results in amylose formation in a restricted zone of the granules. The size of this zone is suggested to be dependent on the GBSS protein level. During development of the granules, the GBSS protein is thought to become limiting, resulting in the formation of starch that lacks amylose. Since the antisense technique conducted in potato utilized a full-length GBSSI cDNA from cassava (~2.2 kb), it has therefore been hypothesized that a small fragment of cassava GBSSI cDNA (<300 bp) that was used for the preparation of RNAi construct might not be effective at suppressing endogenous GBSSI gene in potato. To further determine whether a cassava GBSSI-RNAi construct could be used to suppress the expression of GBSSI gene in potato, more experiments utilizing different fragment size and/or different region of the cassava GBSSI cDNA will need to be conducted.

CHAPTER VI
CONCLUSION

The objectives of this study were to study the effect of RNA interference on the suppression of a target gene in cassava and to investigate the gene expression ability of a cassava tissue-specific promoter in potato. The results obtained from this study could be summarized as follows:

1. Cassava tissue culture and genetic transformation system

1.1 Based on the negative effect of antibiotics, particularly kanamycin and hygromycin B, on the cassava shoot regeneration, the utilization of antibiotics as selective agent was omitted and selection of putative transgenic lines was performed using PCR screening.

1.2 Induction of *in vitro* storage roots of cassava cultivar KU50 was performed and the *in vitro* storage roots could be obtained within 1 to 3 months. This system could be used to study the effect of genes related to starch biosynthesis in controlled environmental conditions.

1.3 The development of friable callus from somatic embryo cluster was established in cassava cultivar KU50. Since the somatic embryo cluster was observed to store starch granules, this system could be used in functional study of genes involved in starch biosynthesis pathway.

2. Potato tissue culture and genetic transformation system

2.1 A combination of cefotaxime at 300 mg/l and hygromycin B at 10 mg/l was preferable when selecting for transgenic potato transformed using *Agrobacterium*-mediated transformation method.

2.2 *In vitro* mini-tubers of potato could be obtained within 2 months. Similar to the *in vitro* storage roots of cassava, the *in vitro* mini-tubers of potato could be used to study the function of genes involved in starch biosynthesis pathway.

3. Investigation of the function of phloem-specific promoter in potato and tobacco

The gene expression ability of phloem-specific promoter p54 from cassava was studied in potato and tobacco. The plasmid pCAMBIA1301::p54::*gus* was transformed into potato via *Agrobacterium* and tobacco via particle bombardment. Even though the transcript of hygromycin resistance gene could be observed by RT-PCR, both potato and tobacco transgenic lines did not show any GUS activity in histochemical localization assay. To investigate the function of phloem-specific promoter in driving the expression of cassava GBSSI-RNAi gene construct, the plasmid pCAMBIA1301::p54::GBSSi::*gus* was transformed into tobacco. The results confirmed that the phloem-specific promoter p54 from cassava was inactive in potato and tobacco.

4. Suppression of GBSSI gene expression in cassava by RNA interference

4.1 Various parameters including size of gold particle, amount of DNA, gas pressure, target distance, number of bombardment, pre- and post-plasmolysed time were examined to determine the effect on transformation efficiency. The appropriate conditions for particle bombardment of cassava would be 1,350 psi acceleration pressure, 6 or 9 cm target distance, 0.6 μm of gold particle size and 20 hr of pre- and post-plasmolysed time.

4.2 By transforming cassava with GBSSI-RNAi construct, three transgenic lines could be obtained. The suppression of GBSSI gene expression in these transgenic lines was supported by RT-PCR analysis and the presence of GBSSI-RNAi fragment in the cassava genome was confirmed by Southern blot hybridization. However, the results indicated that copy numbers of GBSSI-RNAi transgene was not correlated with the degree of GBSSI suppression.

4.3 The cassava transgenic lines carrying GBSSI-RNAi fragment were induced for *in vitro* storage root formation. The *in vitro* storage roots were then subjected to iodine staining. It was observed that the roots from strongest silencing lines were stained brownish, while the wild-type roots were stained blue. This evidence indicated that the amylose/amylopectin ratio in the *in vitro* storage roots of cassava transgenic line was significantly altered. This alteration probably results from the suppression of endogenous GBSSI gene via RNAi mechanism; however, further investigation into this matter needs to be performed.

5. Suppression of GBSSI gene in potato by RNA interference

Transformation of potato with *Agrobacterium* carrying pART27::35S::GBSSi vector yielded two transgenic lines. Although the presence of GBSSI-RNAi transgene could be observed, the *in vitro* mini-tuners from these transgenic lines were stained dark blue with iodine. This result suggested that the GBSSI-RNAi construct prepared using a fragment of GBSSI gene from cassava was not effective in the suppression of potato GBSSI gene. To solve this problem, different regions of cassava GBSSI gene might be used for the preparation of GBSSI-RNAi construct.

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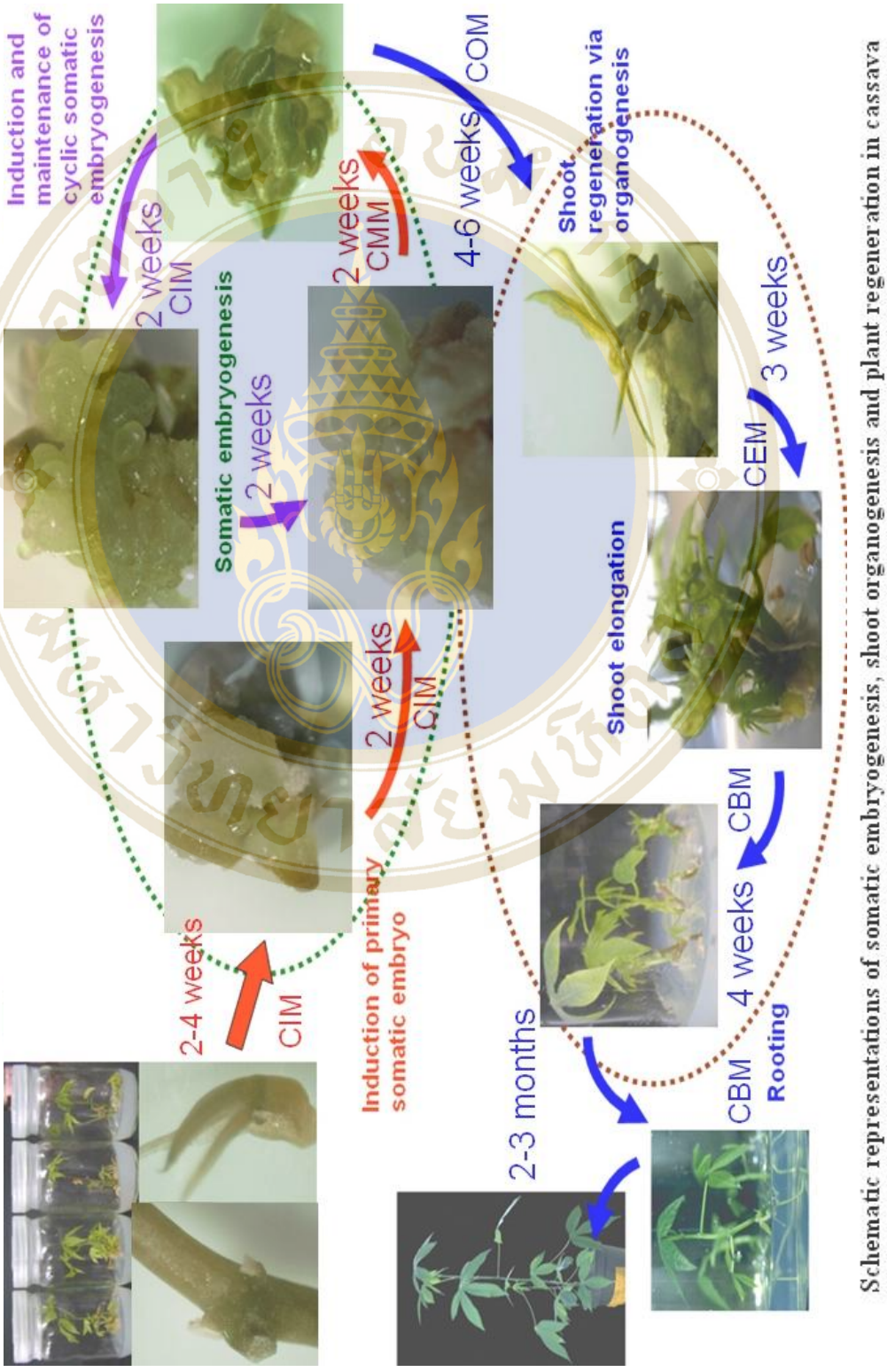
APPENDIX

APPENDIX A



Schematic representations of somatic embryogenesis, shoot organogenesis and plant regeneration in cassava

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** (per liter)

Peptone	10 g
Yeast extracts	5 g
NaCl	10 g
Bacto agar	15 g

3. **MS medium (Murashige&Skoog)** (per liter)

Macronutrients

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

Micronutrients

H_3BO_3	6.2 mg
$MnSO_4.4H_2O$	22.3 mg
$ZnSO_4.7H_2O$	8.6 mg
KI	0.83 mg
$Na_2MoO_4.2H_2O$	0.25 mg
$CuSO_4.5H_2O$	0.025 mg
$CoCl_2.6H_2O$	0.025 mg

Iron

$FeSO_4.7H_2O$	27.85 mg
$Na_2EDTA.2H_2O$	37.25 mg

Organic components

Glycine	2.0 mg
Nicotinic acid	0.5 mg
Pyridoxine	0.5 mg
Thiamine	0.1 mg

4. RMOP medium (tobacco medium) (per liter)

MS basal salts mixture (Phytotechnology)	4.33 g
N6-benzyladenine (BA)	1 mg
1-naphthaleneacetic (NAA)	0.1 mg
Thiamine	1 mg
Inositol	100 mg
Sucrose	30 g
Gelrite	3 g

BIOGRAPHY

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PRESENTATION

Saelim, L., & Narangajavana, J. (2005, November 4-5). *Efficient production of in vitro cyclic somatic embryogenesis and regeneration of the Thai cultivars of cassava (Manihot esculenta Crantz.)* Poster presented at the 3rd International Conference on Starch Technology in BioThailand, Queen Sirikit convention center, Bangkok, Thailand

PUBLICATION

Saelim, L., Phansiri, S., Netrphan, S., Suksangpanomrung, M., & Narangajavana, J. (2006). Optimization of *in vitro* cyclic somatic embryogenesis and regeneration of the Asian cultivars of cassava (*Manihot esculenta* Crantz) for genetic manipulation system. *Global Journal of Biotechnology & Biochemistry, 1*, 7-15.