A Study on *Agrobacterium tumefaciens*-Mediated Gene Transformation in Rubber Tree (*Hevea brasiliensis* Muell. Arg.)

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I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การศึกษาการปลูกถ่ายยีนเข้าสู่ยางพาราโดยใช้อะโกรแบคทีเรียม
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บทคัดย่อ

ยางพารา เป็นพืชที่มีความสำคัญทางเศรษฐกิจของประเทศไทย และจัดอยู่ในสกุล *Hevea* ความสำเร็จในการเพาะเลี้ ยงเนื้อเยื่อที่มีประสิทธิภาพมีบทบาท และความสำคัญต่อการปรับปรุงพันธุ์โดยการปลูกถ่ายยีน ปัจจัยที่มีผลต่อการงอก ของคัพภะ และการชักนำให้เกิดการสร้างยอดรวมของยางพาราในหลอดทดลอง ได้แก่ สารควบคุมการเจริญเติบโต สภาพการวางเลี้ยง และวัสดุพืชที่ใช้ในการเพาะเลี้ยง เริ่มจากการนำคัพภะที่สุกแก่ ที่มีเอ็นโดสเปิร์มมาเพาะเลี้ยงในอาหารสูตร MS เติม BA เข้มข้น 10 มิลลิกรัมต่อลิตร และ IAA เข้มข้น 1 มิลลิกรัมต่อลิตร ให้ อัตราการงอกสูงสุดที่ 93.3 เปอร์เซ็นต์ จากนั้นนำ ชิ้นส่วนยอดเพาะเลี้ยงในอาหารเติม BA เข้มข้น 5 มิลลิกรัมต่อลิตร และ IBA เข้มข้น 1 มิลลิกรัมต่อลิตร ให้จำนวนยอด รวมสูงสุด 4.67 ยอดต่อชิ้นส่วน นอกจากนี้การวาง ชิ้นส่วนข้อที่มีสองตาใน แนวนอนบนอาหาร เพาะเลี้ยง ที่ไม่เติมผงถ่าน ให้การสร้างยอดรวมสูงสุด 2.3 ยอดต่อชิ้นส่วน และจำนวนใบ 5.33 ใบต่อชิ้นส่วน

การปลูกถ่ายยีนที่ต้านทานต่อสารกำจัดวัชพืชไกลโฟเสทเข้าสู่พืชในหลอดทดลองโดยการใช้เชื้ออะโกรแบคเรียม สายเชื้อ AGL-1 ที่มีพลาสมิด pCAMBIA1304 ซึ่งมียีน *gus* เป็นยีนรายงานผล และยีน *EPSPs* ยีนคัดเลือก เริ่มจากการนำยอดบ่มร่วมกับเชื้อที่ความหนาแน่นของค่าความดูดกลืนแสง 600 เท่ากับ 0.6 เป็นเวลา 30 นาทีให้อัตราการรอดชีวิตของชิ้นส่วนสูงสุดบนอาหารคัดเลือก หลังจากการเพาะเลี้ยงเป็นเวลา 45 วัน เมื่อตรวจสอบประสิทธิภาพการถ่ายยีน พบว่ามีการแสดงออกของยีน *gus* และมีการปรากฏแ ถบดีเอ็นเอของยีน *gus* และยีน *EPSPs* โดยใช้เทคนิคพีซีอาร์ dot blot hybridization และ Southern PCR hybridization ซึ่งตำแหน่งของยีน *gus* และ *EPEPs* มีขนาด 919 และ 1,600 คู่เบส ตามลำดับ

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ABSTRACT

Rubber tree is an economical importance in Thailand that belongs to the genus *Hevea.* The success of tissue culture technique plays an importance role for gene transformation in rubber tree. The factors affecting germination of zygotic embryo and multiple shoot formation, including plant growth regulators, culture conditions and culture vessels were evaluated. Mature zygotic embryos with endosperm cultured on MS medium supplemented with 10 mg/L BA and 1 mg/L IAA under light condition for 13 days gave the highest percentage of germination at 93.3. For multiple shoot induction, shoot tip cultured on 5 mg/L BA and 1 mg/L IBA containing medium gave the best result in number of shoots at 4.67 shoots per explant after 40 days of culture. For explant orientation, the result showed that placing 2 nodes horizontally on surface of shoot induction medium without activated charcoal gave the best number of shoots at 2.3 shoots per explants and leaves number at 5.33 leaves per shoot.

To improve it agronomical trait for glyphosate-resistant *in vitro* gene transformation through *Agrobacterium* was conducted. The bacteria carrying plasmid pCAMBIA 1304, harboring *gus* as screenable marker genes and *EPSPs* gene was used. The shoot immersed in *A. tumefaciens* suspension at optical densities (OD_{600}) nm at 0.6 for 30 min gave the higher survival rate after being cultured on glyphosate containing MS medium for one and half months. Assessment of transformed shoots revealed positive results in GUS histochemical assay. The presence of the *gus* and *EPSPs* genes in transformed rubber tree were confirmed by PCR technique, dot blot hybridization and Southern PCR hybridization. Specific primers for the *gus* and *EPEPs* genes were designed to amplify a 919 and 1,600 bps DNA fragment, respectively.

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LIST OF ABBREVIATIONS AND SYMBOLS

AC	=	Activated charcoal
ANOVA	=	Analysis of variance
AS	=	Acetosyringone
BA	=	6-benzyladenine
bar	=	Phosphinothricin acetyltransferase
bp	=	Base pair
CIS	=	*Cis* prenyltransferase
CRD	=	Completely random design
CTAB	=	Hexadecyltrimethylammonium bromide
2,4-D	=	2,4-Dichlorophenoxyacetic acid
DMRT	=	Duncan's multiple range test
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide triphosphate
EC	=	Embryogenic call
EDTA	=	Ethylenediaminetetraacetic acid
EPSPs	=	5-Enolpyruvylshikimate 3-phosphate synthase gene
gfp	=	Green fluorescent protein gene
GFP	=	Green fluorescent protein
gus	=	β-glucuronidase gene
GUS	=	β-glucuronidase
HCl	=	Hydrochloric acid
HMGR	=	Hydroxymethylglutaryl-coA reductase
HMGS	=	Hydroxymethylglutaryl-coA synthase
hpt	=	Hygromycin phosphotransferase
IAA	=	Indole acetic acid
IBA	=	Indolebutyric acid
KN	=	Kinetin

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

KOH	=	Potassium hydroxide
L	=	Litre
LB	=	Lysogeny Broth (medium)
LB	=	Left border
M	=	Molar
ml	=	Milliliter
mm	=	Millimeter
mM	=	Millimolar
mg/L	=	Milligram per liter
MS	=	Murashige and Skoog (medium)
N	=	Normal
NAA	=	α- naphthalene acetic acid
$NaCl_2$	=	Sodium chloride
Na_2EDTA	=	Sodium ethylenediaminetetraacetate
*npt*II	=	Neomycin phosphotransferase
NR	=	Natural rubber
OD	=	Optical density
pat	=	Phosphinothricin acetyltransferase
PCR	=	Polymarese chain reaction
PVP	=	Polyvinyl pyrrolidone
REF	=	Rubber elongation factor
RB	=	Right border
SAS	=	Statistical analysis software
SD	=	Standard deviation
SIM	=	Shoot induction medium
SRPP	=	Small rubber particle protein
TPD	=	Tapping panel dryness

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

TE	=	Tris EDTA
TAE	=	Tris-acetic acid-disodium ethylenediaminetetraacetic acid
TBE	=	Tris-boric acid- disodium ethylenediaminetetraacetic acid
Tris	=	Tris (hydroxymethyl) aminomethane
v/v	=	Volume per volume
w/v	=	Weight per volume
µl	=	Microlitter
µmol m^{-2} s^{-1}	=	Micromole per square meter per second
µM	=	Micromolar

LIST OF PAPERS AND PROCEEDINGS

1. Kalawong, S. and Te-chato, S. 2014. *In vitro* shoot induction of rubber tree using microcutting technique. Songklanakarin Journal of Plant Science 3: 13-19.
2. Kalawong, S., Srichuay W., Sirisom Y. and Te-chato S. 2014. The Establishment of *Agrobacterium*-Mediated Gene Transformation in Rubber Tree through Organized Explants. Journal of Agricultural Technology 10: 493-503.
3. Kalawong, S., Srichuay W. and Te-chato S. 2014. The effect of *Agrobacterium* densities and inoculation time on gene transformation efficiency in rubber tree. African Journal of Biotechnology 13: 2321-2329.

REPRINT PERMISSION

SUMMARY OF CONTENTS

CHAPTER I

General Introduction

General Introduction

1.1 Background

Rubber (*Hevea brasiliensis* Muell. Arg.) clones are intensively cultivated in south-east Asia of the world as main sources for natural rubber (NR) production which is one of the important raw materials for many industries. Laticiferous cells are plant cells producing latex and more than 12,500 plant species have been reported to make these cells. Rubber tree is the main worldwide source of latex to produce commercial natural rubber (Hagel *et al.,* 2008). Natural rubber of rubber tree is considered a raw material by developed countries and is treasure for its high performance properties. Synthetic rubber, consequened from petroleum is not as elastic and does not have the heat transfer properties in comparison with natural rubber. Therefore, natural rubber cannot be easily replaced by synthetically produced polymers. Accordingly, rubber tree is one of the most commercial sources, and the financial records for 42% of the rubber consumed worldwide (Lardet *et al.,* 2011). In breeding and selection methods of any crop species, one of the most significant parameters, bring together character of agronomical interest. Rubber tree is a heterozygous nature that requires grafting using buds as scions on seedling rootstocks for clonal propagation (Cle´ment-Demange *et al.*, 2007). However, breeding of rubber tree, the heterogamous species, is generally taken long time (up to 25 years) and required prolong period for evaluation of mature traits (6-7 years before latex collection). Thus, there are strong limitations for conventional breeding and selection methods (Lespinasse *et al.,* 2000).

So far, the progress of procedures for *in vitro* culture and genetic engineering has been developed. By this procedures, it open a wide possibility in producing rubber genotypes with improved latex yield, tolerance to tapping panel dryness

syndrome, growth rate and wood quality or reduction in undesirable traits (Venkatachalam *et al.,* 2007).

1.2 Rubber tree

Hevea brasiliensis, the principal source of natural rubber, is an open pollinated plant belonging to family Euphorbiaceae. This is intensively cultivated in South East Asia. The main produces of natural rubber in the world are Thailand, Indonesia, Malaysia, India, Vietnam, China, Ivory Coast and Sri Lanka, in descending order of production (Rubber Statistical Bulletin, 2007). Thailand is the world leading producer and exporter of rubber with production capacity of 88-90 percent of total production capacity exported to foreign markets (3.1-3.2 million tons per year) (Saengruksawong *et al.,* 2012). The low heat buildup, higher strength, better resistance to wear and flex cracking make natural rubber a suitable raw material for the product of heavy duty automobile tyres. The high performance properties of natural rubber cannot be easily replaced by synthetically produced polymers. Therefore, the global demand for natural rubber is steadily increasing and hence the production of rubber needs to be increased to meet the demand (Nayanakantha and Seneviratne, 2007). At the moment, the rubber tree cultivar RRIM600 is the main cultivated variety of rubber plantation in Thailand (~70-75%). It is believed that almost all of the other early found clones have been gradually lost. A circumstance means that currently, there is probably a high level of inbreeding clones used as rootstock (Nakkanong *et al.,* 2008). Genetic improvement of rubber tree has been very slow and time-consuming as the major limitations because of narrow genetic base, non-synchronous flowering, low fruit set, long gestation period, heterozygous nature, insufficient availability of land for field experiments and the absence of fully reliable early selection parameters (Venkatachalam *et al.,* 2007). Moreover, many of the elite *Hevea* clones are susceptible to

one or more of undesirable characters such as the physiological disorder as trapping panel dryness and some important diseases especially white root disease, red root disease and phytophthora leaf fall. Before stepping to genetic transformation, a reproducible plant regeneration system for each genotype of *Hevea* through tissue culture techniques are preliminary investigated.

1.3 Tissue culture approaches

Most of the *in vitro* culture work in rubber tree is directed towards micropropagation through nodal cultures, shoot tip culture, and somatic embryogenesis. The first known work on *in vitro* culture of *Hevea* was carried out by Bouychou (1953) of the Institut Francais Caoutchouc, with the aim of using calli to obtain convenient material for the study of the laticeferous system. Micropropagation of rubber tree could be divided into two methods including microcutting and somatic embryogenesis.

Microcutting technique begins by culturing axillary buds or cotyledonary nodes and then inducing plantlets from them. Paranjothy and Gandhimathi (1976) have attempted shoot tip (2-3 cm long) culture, derived from aseptically grown seedlings, for the first time. Although these shoots could be rooted in liquid MS (Murashige and Skoog, 1962) medium, they failed to grow on solid medium. Enjalric and Carron (1982), using shoots derived from 1-3 year old greenhouse grown seedlings as explants, developed rooted plantlets. Thereafter, complete plantlets with shoots and roots could be successfully developed by different investigators. Te-chato and Muangkaewngam (1992) induced multiple shoot from nodal culture of *in vitro* seedling of rubber, landrace cultivars, GT1 and PB5/51. Their results showed that MS medium with BA (6-benzyladenine) alone at concentration of 4.5-5.63 mg/l gave 100% multiple shoot induction and the mean numbers of shoots per explant obtained from GT1, PB5/51 and local cultivar were 3.33 and 3.00, respectively. Root induction was obtained from MS medium with IBA (Indolebutyric acid) in

combination with NAA (α-Naphthaleneacetic acid). Although micropropagation of clonal *Hevea* using axillary shoot proliferation has been achieved to a progressive level (Senevirathne, 1991), there are a number of drawbacks in this system. A single plant could be produced from a single nodal explant and the plants produced by this protocol lack of tap root which is undesirable in clonal tree propagation. Attempts to develop somatic embryogenesis as an *in vitro* propagation technique were started.

Somatic embryogenesis is a rapid and efficient vegetative propagation method. The first anther derived callus which could be subcultured was produced at the Rubber Research Institute of Sri Lanka in 1972 (Satchuthananthavale and Irugalbandara, 1972). Later, this line of work was followed by the Chinese and Malaysian teams. Paranjothy (1974) achieved differentiation of embryoids from anther wall derived callus for the first time. In addition, plant regeneration via somatic embryogenesis in *H. brasiliensis* has been reported using several explants such as immature anther (Jayasree *et al.,* 1999), inner integument of seed (Te-chato and Chartikul, 1993; Montoro *et al.,* 2003; Lardet *et al.,* 2007), unpolinated ovules (Kouassi *et al.,* 2008), immature inflorescence (Sushamakumari *et al.,* 2000) and root (Zhou *et al.,* 2010). The powerful explants were appeared to play important role in the induction somatic embryogenesis including immature anther and inner integument.

The success of embryoids and plant development via somatic embryogenesis was also achieved from anther wall-derived calli (Wang *et al.,* 1984) which is somatic tissue. Jayasree *et al.* (1999) reported the optimized protocol for friable embryogenic callus induction, somatic embryogenesis and plant regeneration from the immature anthers. Optimum callus induction was obtained in modified MS medium supplemented with 2.0 mg/l 2,4-D (2, 4-Dichlorophenoxybenzoic acid) and 0.5 mg/l KN (Kinetin). Somatic embryo induction was found to be better in culture medium containing 0.7 mg/l KN and 0.2 mg/l NAA. Development of the embryos into plantlets was achieved on a

hormone free medium. Cytological analysis revealed that all the plantlets tested were diploid. Subsequently, Hua *et al.* (2010) established self–rooting clones from immature anthers through the following three steps: induction of primary embryos, embryo multiplication by secondary somatic embryogenesis in three successive cycles from single culture of primary embryo and plant regeneration. Optimum plantlet regeneration at 85% was obtained in modified MS medium supplemented with 4.5-13.5 µM 2, 4-D.

For inner integument culture, immature seeds was chosen as explant for developing somatic embryogenesis through four successive phases: (1) callogenesis (2) differentiation of embryos (3) multiplication of embryos and (4) germination of embryos into plantlets (Carron and Enjalric, 1982). Te-chato and Chartikul (1993) successfully induced embryogenic callus and plantlets regeneration from inner integument cultures of immature fruit (8 weeks after pollination) on modified MS medium supplemented with 2 mg/l 2,4-D, 2 mg/l BA, 5-6% sucrose and adjusted pH to 5.6-5.8. Maturation and germination of these embryoids were promoted by excision of each embryoids and transfer to half strength liquid MS medium supplemented with 0.06 mg/l NAA and 0.03 mg/l BA overlaid on activated charcoal supplemented MS agar medium. Sushamakumari *et al*. (2000) developed a technique for somatic embryogenesis and plant regeneration using immature inflorescence explants. They also studied the role of sucrose and abscisic acid (ABA) on embryo induction. A higher sucrose level was found to be essential for effective embryo induction as well as maturation. However, lower concentrations were found to be beneficial for plant regeneration. Repetitive somatic embrogenesis was also induced from primary somatic embryos derived from integumental tissue. Somatic embryos cultured on B-5 medium supplemented with 0.5 mg/l NAA, 2.0 mg/l KN, 0.5 mg/l IAA (Indoleacetic acid) and 4.0 mg/l 2,4-D enhanced repetitive somatic embryogenesis and 5% sucrose was found to be optimum (Asokan *et al.,* 2001). Embryogenic cultures had been maintained for over three

years for retaining the embryo induction and plant regeneration potential (Jayasree and Thulaseedharan, 2001).

For cell suspension technique, initiation and growth of *Hevea* cell suspension cultures have also been evaluated. Suspension culture offers advantages of mass propagation *in vitro*, particularly for somatic embryogenesis. Callus derived from stem segment cultures on semi-solid medium failed to yield a good cell suspension when transferred to agitate in modified MS liquid medium (Wilson and Street, 1975). Studies have also been made to determine the effect of photoperiod on growth and differentiation of anther wall callus (RRIM, 1981). Te-chato and Chartikul (1993) conducted cell suspension and embryogenic suspension of rubber tree by dispersion of integument-derived calli in various liquid culture medium. Among culture media tested, MS medium with 0.5 mg/L 2,4-D and 1.0 mg/L BA was the best for induction and maintenance of the suspension.

Recently, there has been a renewed interest in *Hevea* for the development of techniques for plant regeneration through somatic embryogenesis, especially for use in genetic transformation (Jayasree *et al.,* 1999).

1.4 Genetic engineering

Conventional breeding of rubber takes more than 25 years to achieve a new clone. Genetic engineering offers a potential way to breeders for adding desirable traits to crop plants, leading to the progress of elite clones in a short time. The rubber tree is a good candidate for handling by genetic transformation because of long breeding cycle and heterozygous nature. In addition, tissue culture techniques including plant regeneration via microcutting or somatic embryogenesis have been considered. Several agronomic traits that could be established for rubber improvement through genetic engineering program include TPD (tapping panel dry) tolerance, high yield potential, resistance to diseases, production of recombinant protein and timber wood quality improvement. Genes expressed

in the latex of *Hevea* can be divided into three groups based on the proteins they encode: rubber biosynthesis- related proteins such as REF (rubber elongation factor), HMGR (hydroxymethylglutaryl-coA reductase), HMGS (hydroxymethylglutaryl-coA synthase), CIS (*cis*prenyltransferase), GGPP (geranylgeranyl diphosphate) synthase, SRPP (small rubber particle protein), IPP (isopentenyl diphosphate) isomerase; defense/stress-related proteins such as MnSOD, hevein, chitinase, β-1,3-glucanase and HEVER; and latex allergen proteins such as Hev.b.3, Hev.b.4, Hev.b.5, Hev.b.7. Biological functions of the allergenic proteins are largely unknown.

1.5 The history of *Agrobacterium*-mediated genetic transformation in rubber tree research

Genetic engineering of crop plants integrated with specific genes has been achieved either by direct gene transfer or by *Agrobacterium tumefaciens* mediated genetic transformation. *A. tumefaciens*-mediated genetic transformation technique has most widely been used for plant species due to its easy protocol without any special equipment. These technique were developed on numerous *Hevea* clones, GL1, RRII 105 and PB 260 (Arokiaraj *et al.,* 1994; Jayashree *et al.,* 2003; Priya *et al.,* 2006; Montoro *et al.,* 2003; Lardet *et al.,* 2011). Montoro *et al.* (2003) developed genetic transformation protocol using *A. tumefaciens* in friable integument callus line (clone PB260) for a good frequency of integration of transgenic calli. An efficient genetic transformation procedure was also investigated using highly integument-callus lines (Blanc *et al.,* 2006) and GFP selection of transformants (Leclercq *et al.,* 2010). Nowadays, over-expression of endogenous genes involved in reactive oxygen species scavenging systems, such as MnSOD has been reported (Jayashree *et al,.* 2003). The target of transgene expression in latex cells was also attempted using the promoter from gene *HEV*2.1, which was the major latex Hevein protein (Montoro *et al.,* 2008). Moreover, genetic transformation protocol was developed using the

transfer of a synthetic *EPSPs* (5-Enolpyruvylshikimate-3-phosphate synthase gene) transgene, as a conditional positive selectable marker, into commercially relevant zonal pelargoniums using an *A. tumefaciens* strain in combination with a novel step-down glyphosate selection system. Glyphosate is a commercial herbicide used in control of weed species which exerts its action on plants through inhibition of *EPSPs*. This chemical is not detoxified, and consequently here is no cross-protection afforded to evolve resistant weeds (Chen *et al.,* 2012). In addition, glyphosate is a highly mobile selection agent and translocates throughout the plant and so is less dependent on direct contact of the target tissue than some other selection agents (Howe *et al.,* 2002). However, transformation efficiency was depended on many factors, protocols and cultivar-dependent. So far, there has not been a suitable common platform of genetic transformation method for all kinds of *Hevea*. Accordingly, there are still many problems to be solved in establishing a high efficient and stable genetic transformation system of *Hevea*. Thus, it is necessary to deeply study the factors affecting the *Hevea* transformation frequency.

1.6 Factors affecting *Agrobacterium*-mediated genetic transformation in *Hevea*

The genetic transformation as a key technology for genetic engineering of rubber tissue has made a great improvement. At the moment, the rapid, stable, high efficient and large-scale genetic transformation system in most *Hevea* was established. Influencing factors of high efficiency *Hevea* genetic transformation system mediated *Agrobacterium,* include antibiotic compounds (cefotacime or not antibiotic), time of infection, time of co-culture and bacterial cell density.

1.6.1 Antibiotics

An antibiotic is an chemical agent that either kills or inhibits the growth of a microorganism. Antibiotics such as cefotaxime, carbenicillin and timentin have been used

regularly in *Agrobacterium*-mediated transformation of crops following co-culture to suppress or eliminate bacteria. Nauerby *et al.* (1997), who reported that 500 mg/L cefotaxime and 1000 mg/L carbenicillin played an inhibitory effect on regeneration of *Nicotiana tabacum*. Pipatpanukul *et al.* (2004) tested the effect of antibiotics on the regeneration of rice calli. Antibiotics strongly decreased regeneration capacities of calli. The highest dose of both carbenicillin and cefotaxime (up to 250 mg/L) completely inhibited plant regeneration (phytotoxicity). Carbenicillin at 100 mg/L was applied for all the subsequent experiments, and it has been the antibiotic of alternative reports of *Agrobacterium*-mediated transformation of wheat and maize (Zheng *et al.,* 2001).

Concentrations of selective agents or antibiotics are needed to avoid development of undesirable escape numbers. Cheng *et al.* (1997) reported the presence of 50 mg/L hygromycin throughout callus as regeneration period to avoid development of the escapes. Alternatively, 100 mg/L kanamycin was economical and improved the transformation efficiency in white spruce by enrichment of transformed tissue in bud forming callus (Le *et al.,* 2001) and increased the proportion of positively transformed shoots during subculture on kanamycin containing medium in peanut and pigeon pea (Thu *et al.,* 2003). Yang *et al.* (2010) reported the use of various concentrations of kanamycin to establish a suitable level for the selective growth for possible selection of transformed plant cells against the non-transformed background. At 100 mg/L of kanamycin significantly inhibited the growth of embryogenic callus, and severely at higher than this concentrations. However, calli cultured on the media with 200 mg/L kanamycin or higher became white and gradually died within 30 days, while the calli cultured on the control medium grew vigorously. Finally, kanamycin at concentration of 200 mg/L was used to discriminate between transformed and non-transformed cells.

In case of selection transformed calli or plantlets by glyphosate, a complete protocol of *in vitro* selection and greenhouse screening for glyphosate-tolerant variants in

transgenic plant was established. Chen *et al.* (2012) reported newly transgenic manila grass calli more than 5 years' old were transferred to selection medium with 2 mM glyphosate. Plantlet regeneration from transgenic grasses was again transferred to regeneration medium with 0.5 mM glyphosate to selected toleraned plantlets. However, various sources of explants used resisted to different concentrations of glyphosate. Boase *et al.* (2012) studied concentrations of glyphosate that inhibit all shoot regeneration from culturing explants. The number of explants regenerating shoots decreased from 100% (0 µM of glyphosate) to 0% (120 and 150 µM of glyphosate). Therefore, the 150 µM glyphosate concentration was used in selection medium for 42 days to provide a buffer against shoots escaping selection.

1.6.2 Co-culture conditions

Gene transformation using *Agrobacterium tumefaciens* consisted of many steps such as bacteria attachment, *vir* gene induction, T-DNA processing, T-DNA transportation and T-DNA integration, which completed co-cultivation time (Yang *et al.,* 2011). Therefore, the main factor affecting successful transformation was co-cultivation. The efficiency of co-cultivation was affected by the gene transferred chemical AS (acetosyringone), bacterial cell density, infection time and co-cultivation time.

Acetosyringon was recommended for *vir* gene induction in most of the mococotyledonous plant transformation protocols (Zhao *et al.,* 2000). The AS was an key phenolic compound, naturally produced by wounded plant cells, and activated the *vir* genes of the Ti plasmid and to initiate the transfer of the T-DNA. This chemical was used at a concentration of 100 µmol/L for EC (Embryogenic callus) transformation of wine grape variety Thompson seedless (Fan *et al.,* 2002). Nevertheless, different plant materials and transformation protocols require different concentrations of AS to facilitate the

Agrobacterium-mediated gene transformation. High concentration of AS at 200 µmol/L was reported for gene transformation in orchid (Belarmino and Mii, 2000).

One of the critical factors affecting the final transformation efficiency is the *Agrobacterium* density used for infection. If the bacterial concentration is low, transformation efficiency also low, while very high bacterial concentration is easy for over growth of bacteria. Too much bacteria are difficult in their elimination, leading to plant necrosis and following cells death.

Infection and co-cultivation time were the most important steps in the transformation process and significantly affect the final efficiency of the transformation process. Zhao *et al.,* (2011) compared the different time span of infection and following co-cultivation of *Vitis vinifera* calli. The number of cells with green fluorescence was remarkably higher with 30 min after inoculation with *Agrobacterium*. Co-cultivation calli with *Agrobacterium* for 3 days did not show significant browning and had higher transformation efficiency. Normally, overgrowth of *Agrobacterium* on the surface of explant causes the dark brown of the calli easily leading to stop growing of them.

1.6.3 Selectable marker and molecular assessment

The most widely used selectable markers for transformation of crops were genes encoding *hpt* (hygromycin phosphotransferase), *pat or bar* (phosphinothricin acetyltransferase), *npt*II (neomycin phosphotransferase) and *EPSPs*. These marker genes under the control of constitutive promoters such as the 35S promoter from cauliflower mosaic virus, the ubiquitin promoter from maize, works efficiently for selection of *Agrobacterium*-transformed cells. The *npt*II gene under the control of the nopaline synthase promoter has been used successfully to select stable transformants with kanamycin. Glyphosate insensitive plant containing *EPSPs* genes derived from bacteria that degrades

glyphosate. Among those genes, *EPSPs* has been successfully used in *Agrobacterium* transformation in wheat (Hu *et al.,* 2003). Moreover, genetic transformation protocol was developed using the transfer of a synthetic *EPSPs* transgene, as a conditional positive selectable marker, into commercially relevant zonal pelargoniums using an *A. tumefaciens* strain in combination with a novel step-down glyphosate selection system.

The introduction of foreign genes into plants was assessed in the transformants by PCR analysis and Southern blot hybridizations. The processes of genetic transformation in *H. brasiliensis* using *A. tumefacients* for glyphosate resistant rubber were optimized.

1.7 Objectives

The objectives of this study were to establish tissue culture system in indigenous clone of *Hevea brasiliensis* and transfer of foreign genes using *A. tumefaciens* strain EHA 105 harbouring plasmid pCAMBIA 1301 with *EPSPs* as desirable and *gus* (ß-glucuronidase) as reporter geneglyphosate resistant rubber was optimized. The introduction of foreign genes into plant cell was assessed in the transformants by PCR analysis and Southern blot hybridizations.

CHAPTER II

Preparation of Plant Materials for Gene Transformation

Experiment I

Improvement Tissue Culture Technique of Para Rubber for Gene-Transformation

Introduction

Rubber tree is a heterozygous perennial plant with a long reproductive maturity. This tree is propagated mostly by grafting high producing latex buds on heterogeneity of the rootstocks produced from seeds (Hua *et al.,* 2010). Breeding program takes 25 years to select and recommend a new clone. Accordingly, improvement of this tree via conventional breeding is very difficult and takes so long time (Lardet *et al.,* 2011). In addition, latex yield in rubber plantations has been significantly increased by releasing high latex yielding clones for cultivation over the past decades, an estimated annual rubber production loss due to TPD at 15–20% (Chen *et al.,* 2003). The immediate effect of TPD is the appearance of partial dry zones (no latex flow) and in the advanced stage, the tapping panel may even become completely dry and other symptoms such as browning, thickening, or even flaking of bark can occur (Sookmark *et al.,* 2002). An alternative strategy that may potentially shorten breeding time is genetic engineering. It can be used to produce desirable agronomic traits quickly and efficiently (Arokiaraj *et al.,* 2002). The success of tissue culture technique plays an importance role for gene transformation in rubber tree.

Most of the *in vitro* culture works in rubber tree are directed towards micropropagation through nodal cultures, shoot tip culture, and somatic embryogenesis. Thus, micropropagation of rubber tree could be divided into two methods including microcutting and somatic embryogenesis. For micropropagation true-to-type clones were propagated. This technique is always used for propagation of both rootstock and high yielding clones. Microcutting technique begins by culturing axillary buds or cotyledonary nodes and then inducing plantlets from them. Culture conditions, plant growth regulators and other nutritional requirement for improving the efficiency of shoot induction and shoot formation were investigated by Te-chato and Muangkaewngam (1992). Multiple shoots was induced from nodal culture of *in vitro* seedling of rubber, landrace cultivars, GT1 and

PB5/51. Their results showed that MS medium with BA alone at concentration of 4.5-5.63 mg/l gave 100% multiple shoot induction and the mean numbers of shoots per explant obtained from GT1, PB5/51 and local cultivar were 3.33, 3.00 and 3.00, respectively. Root induction was obtained on MS medium with IBA in combination with NAA. Development of protocols for micropropagation will greatly facilitate production of true-to-type elite planting material eliminating stock-scion interaction leading to intraclonal variation in field performance.

Materials and Methods

Plant materials

Seeds of rubber tree clone Tjir1 grown at Prince of Songkla University, Hatyai campus, Songkhla province, Thailand, were used in this experiment (Figure 2.1a). Seeds without seed coat were washed in running tap water for 10 minutes (Figure 2.1b). The explants were surface sterilized in 70% ethanol for 30 seconds and in 20% sodium hypochlorite for 20 minutes, followed by three rinses with sterilized distilled water.

Effects of plant growth regulators (BA and/or IAA) and culture conditions on seed germination

The sterilized seeds with endosperm were trimmed (Figure 2.1c) and cultured on MS medium supplemented with 0, 5, 10 and 15 mg/L BA and/or 0, 1, 5 and 10 mg/L IAA, 3% sucrose and 0.05% activated charcoal. The medium's pH was adjusted to 5.7 with 0.1 N HCl (hydrochloric acid) or KOH (potassium hydroxide) before adding 0.75% agar and autoclaved at 1.05 kg/cm^2, at 121 ºC for 15 minutes. The cultures were maintained at 28±0.5 ^{o}C under fluorescent lamps at 12.5 µmol/m^2/s for a 14 hour photoperiod. After being cultured for 13 days the frequency of seed germination, root number, root length, shoot number and shoot length of rubber seedling were recorded and statistically

compared. The data were statistically analyzed using completely randomized design (CRD) and the means among the treatments were separated by Dancan's multiple range test (DMRT).

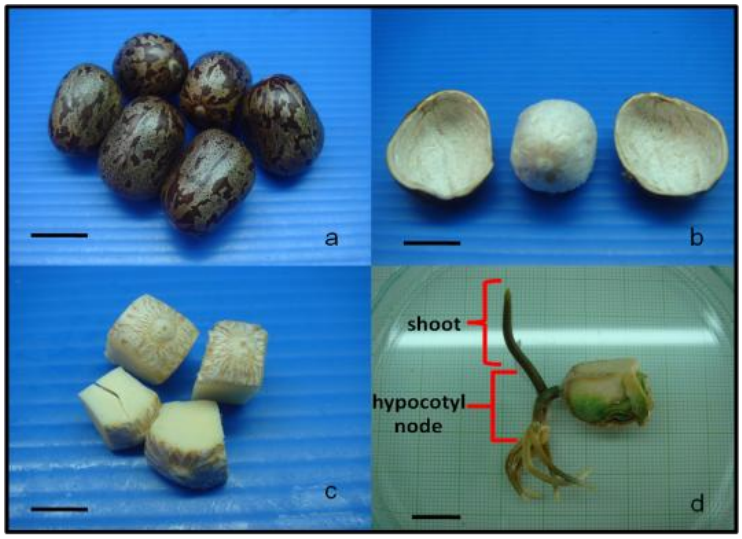

Figure 2.1 Rubber seeds and *in vitro* plantlet of early introduced clone of rubber tree no. 4 grown at Prince of Songkla University.

(a) seeds (bar 1 cm) (b) seeds without seed coat (bar 1 cm)

(c) trimmed seeds with embryos with endosperm (bar 0.5 cm)

(d) *in vitro* plantlet after 13 days of germination (bar 1 cm)

Effects of plant growth regulators (BA and/or IBA) and culture conditions on seed germination

To study the effect of BA and IBA on shoot formation. Plantlets were cut into two pieces which were hypocotyl and epicotyl (Figure 2.1d). These explants were cultured on MS medium supplemented with 0, 5, 10 and 15 mg/L BA and/or 0, 1, 5 and 10 mg/L IBA and 3% sucrose. The medium's pH was adjusted, autoclaved and the cultures were maintained as the same methods as previous experiment. After being cultured for 40 days the frequency of shoot number, leaf number and root number were recorded and

statistically compared. The data were statistically analyzed using CRD and the means among the treatments were separated by DMRT.

Effects of type of explants, activated charcoal and orientation of explants on multiple shoot formation

Single shoot, nodal with a bud and nodal with two buds were obtained from previous study. Three sources of plant material were placed on MS medium supplemented with 5 mg/l BA, 1 mg/L IBA, 3% sucrose and/or 0.05% activated charcoal in two directions (vertical and horizontal directions). The pH of culture medium was adjusted, autoclaved and the cultures were maintained as the same methods as previous experiment. After being cultured for 4 weeks, shoot number, shoot length, leaf number and leaf length were recorded. The data were statistically analyzed using 3x2x2 factorial in CRD and the means among the treatments were separated by DMRT.

Results and Discussion

Factors affecting seed germination, including plants growth regulators and culture conditions were studied. Mature embryos with partial endosperm were cultured on MS medium supplemented with various concentrations of plant growth regulators, including BA (1, 5 and 10 mg/L) and IAA (1 and 3 mg/L) and maintained under 12.5 μmol/m^2/s (14 h photoperiod) illumination or darkness for 13 days. The results showed that the highest percentage of germination at 93.3 was obtained from mature embryo cultured in liquid MS medium supplemented with 10 mg/L BA and 1 mg/L IAA under light condition, significant difference with the other treatments. The characteristics of radicle development after 3 and 5 days of seed germination under light condition were shown in Figure 2.2a, b. After that, hypercotyls and cotyledons were formed (Figure 2.2c, d). However, seed cultured on MS

medium without plant growth regulators gave only 60 percentage of seed germination (Table 2.1). This suggested that different concentrations of plant growth regulators containing MS medium were effective in seed germination of rubber tree. Thus, this seed was immaturity seed. In case of dark condition, plantlet was white in color and elongate (Figure 2.2e-h). For root number, medium with 1-10 mg/L BA and 3 mg/L IAA gave the highest root numbers at 11-12 roots/shoot under dark condition. However, medium without IAA gave high root length around 3-6 cm. For shoot number, all treatment had only 1 shoot. High shoot length were found from 1-10 mg/L BA and 1 mg/L IAA under both light and dark conditions. The advantage of this study will be very useful for *in vitro* micrografting in further experiment.

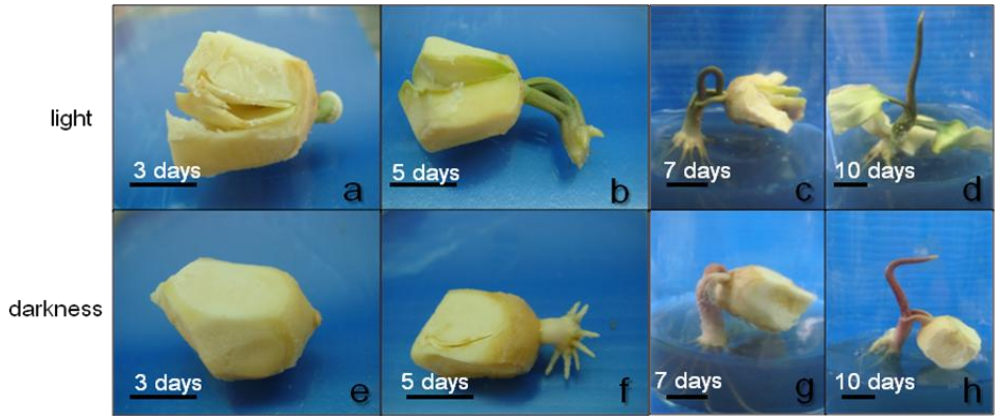

Figure 2.2 Germination of rubber tree seed on medium containing plant growth regulators (BA and/or IAA) and culture conditions (light and darkness) after 10 days of culture (bar=0.3).

Table 2.1 Effects of plant growth regulators (BA and/or IAA) and culture conditions on seed germination, root number, root length, shoot number and shoot length after 13 days of culture.

Culture conditions	BA (mg/L)	IAA (mg/L)	Germination (%)	Root number	Root length (cm)	Shoot number	Shoot length (cm)
Light	0	0	60bcde	4cd	3.23ef	1	8.03cde
	1	0	66.67abcd	4.33cd	5.57abc	1	11.33ab
	5	0	86.67ab	7.33c	5.2abcd	1	8.73abcd
	10	0	80ab	6.67cd	4.77abcde	1	9.6abcd
	1	1	73.33abc	6cd	4.23bcde	1	9.67abcd
	5	1	73.33abc	7.33c	4.27bcde	1	10.33abc
	10	1	93.33a	7cd	3.43def	1	10.2abc
	1	3	80ab	5.33cd	3.13ef	1	8.57bcd
	5	3	93.33a	6.33cd	3.7cdef	1	6.57de
	10	3	46.46cdef	4.67cd	2.07f	1	5.37e
Dark	0	0	33.33ef	4.67cd	3.9cdef	1	8.57bcd
	1	0	36.67ef	3d	6.23a	1	9.17abcd
	5	0	46.67cdef	5cd	5.87ab	1	10abc
	10	0	40def	6cd	5.47abc	1	9.7abcd
	1	1	33.33ef	6cd	4.93abcde	1	10.33abc
	5	1	40def	8cb	5abcde	1	11.8a
	10	1	46.67cdef	7cd	3.9cdef	1	11.27ab
	1	3	33.33ef	11.67a	3.77cdef	1	8.7abcd
	5	3	66.67abcd	11a	4.17bcde	1	8.7abcd
	10	3	26.67f	12a	3.2ef	1	7.5cde
	F-test		**	**	**	ns	**
	C.V. (%)		26.51	30.56	22.89	0	17.58

ns : non significant difference ** : highly significant difference at $P \leq 0.01$

Means followed by the same letter within each column are not significantly different according to DMRT

Attempt to develop multiple shoot induction from shoot apices and hypocotyl nodes were made by culturing those explants on MS medium supplemented with different types and concentrations of plant growth regulators. The results revealed that shoot apices cultured on 5 mg/L BA and 1 mg/L IBA containing culture medium gave the best result in number of shoots at 4.67 shoots per explant (Figure 2.3c) and leaf number at 6.56 leaves after 40 days of culture (Table 2.2), significant different with another plant growth regulators. The use of the shoots and nodes of seedlings as initial explants can be successfully used for *in vitro* multiplication on the shoot induction medium as earlier described by Te-chato and Muangkaewngam (1992). In case of hypocotyl node, 5 mg/L BA and 1 mg/L IBA containing culture medium gave the best result in number of shoots at 1.89 shoots per explants after 40 days of culture. The medium without BA gave high root number around 1 root (in combination with 0 mg/L IBA) to 7 roots (in combination with 1 mg/L IBA) (Table 2.3). Auxin alone gave the best result in promoting root formation from shoot apices and hypocotyls node explants (Figure 2.3b, f).

Effects of types of explants, activated charcoal and orientation of explant on multiple shoot formation and shoot elongation were investigated. The results showed that placing node with 2 buds horizontally on surface of shoot induction medium without activated charcoal gave optimal result in shoot number at 2.3 shoots per explant (Table 2.4), shoot length at 0.26 cm (Table 2.5), leaf number at 5.33 leaves per shoot (Table 2.6) and leaf length at 0.47 cm (Table 2.7). Placing the explants by horizontal orientation promoted the better absorption of nutrition and plant growth regulators by both surface tissues (Figure 2.4). Thus, orientation of both shoot and nodal (1 bud, 2 buds) explants horizontally on culture medium gave better result in multiple shoot formation (1.78 shoots), shoot length (0.27 cm), leaf number (3.17 leaves) and leaf length (0.40 cm) than vertical orientation. In case of source explants, the best result in multiple shoot formation was produced from node with two buds at 1.83 shoots.

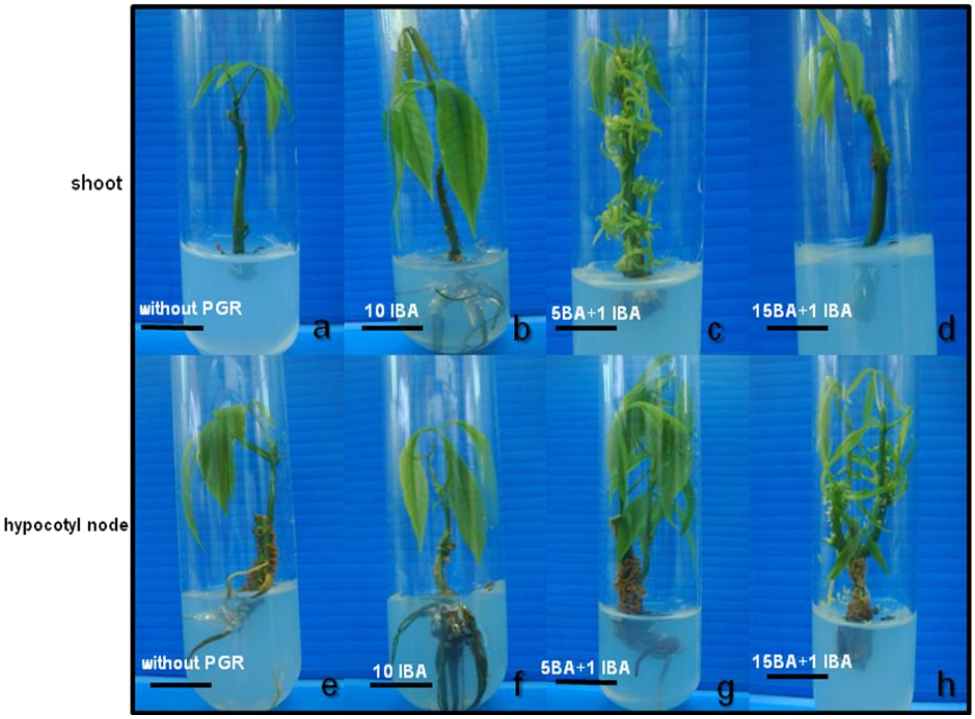

Figure 2.3 The characteristics of multiple shoot formation from shoot and hypocotyl nodal explants on culture medium with or/and with plant growth regulators (BA and/or IBA) after 40 days of culture (bar 0.5 cm).

Table 2.2 Effects of plant growth regulators (BA and/or IBA) on shoot, leaf and root number from culturing shoot tips after 40 days of culture.

BA (mg/L)	IBA (mg/L)	Shoot number	Leaf number	Root number
0	0	1d	2c	0d
	1	1d	1.78c	1.67c
	5	1d	1.44c	7.78a
	10	1d	1.67c	4.67b
5	0	2.33c	1.78c	0d
	1	4.67a	6.56a	0d
	5	3.78b	3.32b	0d
	10	3.22b	2.22c	0d
10	0	0.89d	1.89c	0d
	1	1.11d	1.78c	0d
	5	1.11d	1.78c	0d
	10	0.55d	1.89c	0d
15	0	0.67d	2c	0d
	1	0.56d	1.44c	0d
	5	0.44d	1.56c	0d
	10	0.44d	1.78c	0d
F-test		**	**	**
C.V. (%)		34.23	19.45	48.52

** : highly significant difference at $P \leq 0.01$

Means followed by the same letter within each column are not significantly different according to DMRT

Table 2.3 Effects of plant growth regulators (BA and/or IBA) on shoot, leaf and root number from culturing hypocotyl nodal explants after 40 days of culture.

BA (mg/L)	IBA (mg/L)	Shoot number	Leaf number	Root number
0	0	0.44f	2.45d	1.56d
	1	1.11de	3.33cd	4.22c
	5	1.33cd	2.11d	5.33b
	10	1.22de	2d	7.22a
5	0	0.67ef	2.11d	0e
	1	1.89c	3.66bcd	0.33e
	5	1.11de	2d	0e
	10	1.11de	1.56d	0e
10	0	1.56cd	2.67cd	0e
	1	1.33cd	3cd	0e
	5	2.78b	5.55b	0e
	10	1.44cd	3.61bcd	0e
15	0	1.56cd	5.46b	0e
	1	3.33a	8.55a	0e
	5	1.33cd	4.56bc	0e
	10	1.67cd	3.56bcd	0e
F-test		**	**	**
C.V.(%)		20.41	30.95	35.72

** : highly significant difference at P ≤ 0.01

Means followed by the same letter within each column are not significantly different according to DMRT

Table 2.4 Effects of types of explants, activated charcoal and orientation of explant on multiple shoot formation on shoot induction medium after 1 month of culture.

Explants	Number of shoots				Average ^{explant}
	Vertical orientation		Horizontal orientation		
	+AC	-AC	+AC	- AC	
1 Node	1.00c	1.00c	1.00c	1.3bc	1.08B
2 Node	1.67abc	1.67abc	1.67abc	2.33a	1.83A
Shoot tip	1.00c	1.00c	2.00ab	2.33a	1.58A
Average ^{orientation}	1.22B		1.78A		
Average ^{medium}	1.38		1.61		
F(Rep)	*				
F(Medium)	ns				
F(Explant)	**				
F(Orientation)	**				
F(Medium X Explant)	ns				
F(Medium X Orientation)	ns				
F(Explant X Orientation)	**				
F(Medium X Explant X Orientation)	ns				

C.V. (%) = 27.22 ns : non significant difference

* : significant difference at P ≤ 0.05 ** : highly significant difference at P ≤ 0.01

Means among treatment combinations followed by the same letter within each row and column are not significantly different according to DMRT

Table 2.5 Effects of types of explants, activated charcoal and orientation of explant on shoot length after culture on shoot induction medium for 1 month.

Explants	Shoot length (cm)				Average ^{explant}
	Vertical orientation		Horizontal orientation		
	+AC	- AC	+AC	- AC	
1 Node	0.10d	0.10d	0.23bc	0.20cd	0.16B
2 Node	0.10d	0.17cd	0.26abc	0.26abc	0.20AB
Shoot tip	0.17cd	0.17cd	0.30ab	0.33a	0.24A
Average ^{orientation}	0.13B		0.27A		
Average ^{medium}	0.20		0.19		
F(Rep)	ns				
F(Medium)	ns				
F(Explant)	**				
F(Orientation)	**				
F(Medium X Explant)	ns				
F(Medium X Orientation)	ns				
F(Explant X Orientation)	ns				
F(Medium X Explant X Orientation)	ns				

C.V. (%) = 26.35 ns : non significant difference

** : highly significant difference at P ≤ 0.01

Means among treatment combinations followed by the same letter within each row and column are not significantly different according to DMRT

Table 2.6 Effects of types of explants, activated charcoal and orientation of explant on leaf number after culture on shoot induction medium for 1 month.

Explants	Leaf number				Average explant
	Vertical orientation		Horizontal orientation		
	+AC	- AC	+AC	- AC	
1 Node	0.00d	0.00d	2.00c	2.67bc	1.17b
2 Node	0.00d	0.00d	2.67bc	5.33a	2.00a
Shoot tip	0.00d	0.00d	3.00bc	3.33b	1.58ab
Average orientation	0.00B		3.17A		
Average medium	1.28B		1.89A		
F(Rep)	ns				
F(Medium)	**				
F(Explant)	**				
F(Orientation)	**				
F(Medium X Explant)	*				
F(Medium X Orientation)	**				
F(Explant X Orientation)	**				
F(Medium X Explant X Orientation)	*				

C.V. (%) = 37.95 ns : non significant difference

* : significant different at P ≤ 0.05 ** : highly significant difference at P ≤ 0.01

Means among treatment combinations followed by the same letter within each row and column are not significantly different according to DMRT

Table 2.7 Effects of type of explants, activated charcoal and orientation of explants on leaf length after culturing on shoot induction medium for 1 month.

Explants	Leaf length (cm)				Average explant
	Vertical orientation		Horizontal orientation		
	+AC	- AC	+AC	- AC	
Node (1 bud)	0.00d	0.00d	0.20c	0.23c	0.11B
Node (2 buds)	0.00d	0.00d	0.37bc	0.47ab	0.21A
Shoot	0.00d	0.00d	0.53ab	0.60a	0.28A
Average orientation	0.00B		0.40A		
Average medium	0.21		0.18		
F(Rep)			ns		
F(Medium)			ns		
F(Explant)			**		
F(Orientation)			**		
F(Medium X Explant)			ns		
F(Medium X Orientation)			ns		
F(Explant X Orientation)			**		
F(Medium X Explant X Orientation)			ns		

C.V. (%) = 48.59 ns : non significant difference

** : highly significant different at P ≤ 0.01

Means among treatment combinations followed by the same letter within each row and column are not significantly different according to DMRT

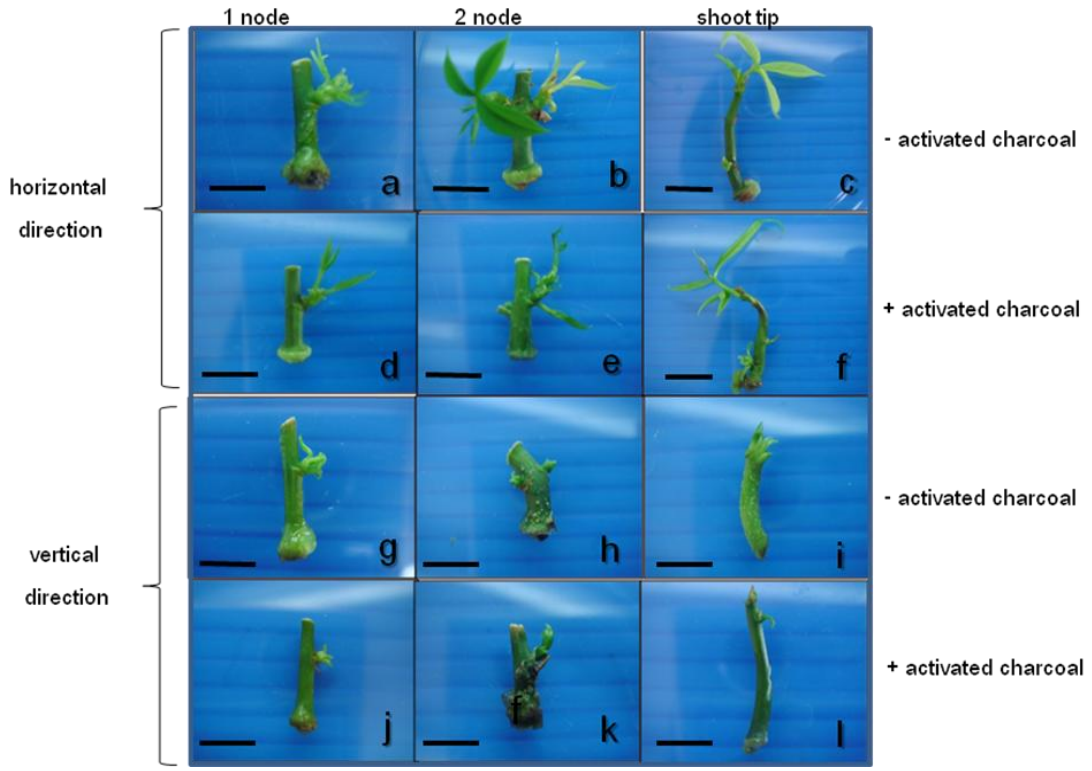

Figure 2.4 The characteristics of new forming shoots from three different explant types with two different orientations on shoot induction medium without or with 0.05% activated charcoal after 1 month of culture (bar 0.2 cm).

CHAPTER III

Assessment of Transformation Efficiency

Experiment I

The Establishment of *Agrobacterium*-Mediated Gene Transformation in Rubber Tree through Organized Explants

Introduction

An alternative strategy that may potentially shorten breeding time is genetic engineering. It can be used to produce desirable agronomic traits quickly and efficiently (Arokiaraj *et al.,* 2002). An efficient *Agrobacterium tumefaciens*-mediated genetic transformation procedure has been developed from friable integument callus line for clone PB260 with a good frequency of integration and with a majority of transgenic calli (Montoro *et al.,* 2003). An efficient genetic transformation procedure using a recombinant *gfp* (green fluorescent protein) has been developed in *H. brasiliensis* clone PB260 (Leclercq *et al.,* 2010). Glyphosate selection has a number of advantages over other commonly used selectable markers. For example, in maize, glyphosate selection can yield a very low frequency of non-transgenic escapes compared to kanamycin, where selection is quite inefficient. Glyphosate is not detoxified, and consequently, there is no cross-protection afforded to adjacent cells. In addition, glyphosate is a highly mobile selection agent and translocates throughout the plant and so is less dependent on direct contact of the target tissue than some other selection agents (Howe *et al.,* 2002). A genetic transformation protocol was developed using the transfer of a synthetic *EPSPs* transgene, as a conditional positive selectable marker, into commercially relevant zonal pelargoniums using an *Agrobacterium tumefaciens* strain in combination with a novel step-down glyphosate selection system. Moreover, the presence of *Agrobacterium* in transformed tissues, even after the use of bacteriostatic antibiotics, can give false-positive PCR (polymarese chain reaction) results (Boase *et al.,* 2012). So, the aim of present study was to improve gene transformation procedure in *H. brasiliensis* using *Agrobacterium* for transferring some important genes in the future.

Materials and Methods

Plant material

Seeds from rubber tree clone Tjir1, naturally grown at Prince of Songkla University, Hatyai campus, Songkhla province, Thailand, were collected and used as explants for zygotic embryo culture. After 2 weeks of culture, seedings were obtained and they were excised into two parts, shoot tip and hypocotyl node. The two explants were cut into 1.5 cm in length and cultured on SIM (shoot induction medium) supplemented with 5 mg/l BA, 3% sucrose and 0.05% activated charcoal as described by Te-chato and Muangkaewngam (1992). The medium's pH was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaved at 1.05 kg/cm^2, at 121ºC for 15 minutes. The cultures were maintained at 28±0.5 $^{\circ}$C under fluorescent lamps at light intensity of 12.5 µmol/m^2/s, 14 hour photoperiod for 1 month.

Bacterial plasmid

A. tumefaciens strain EHA105 containing the plasmid pCAMBIA1304-*EPSPs* which harbored *gus* and *EPSPs* genes (Figure 3.1) was used in this study. A single colony of this bacteria was pick out and suspended in 25 ml liquid LB (Lysogeny Broth) medium (10 g/L tryptone, 5 g/L Bacto yeast extract, 5 g/L NaCl, pH 7.0) containing 50 mg/L kanamycin and incubated on a rotary shaker at 100-150 rpm in the dark at 28ºC. After proliferation in LB medium overnight the cells were collected and adjusted density by spectrophotometer at OD (optical density) of 600 nm at 0.6.

Inoculation and selection of transgenic calli

The shoot tips and nodal segments (0.3-0.5 cm) were immersed in 25 ml the *A. tumefaciens* suspension at OD of 600 nm at 0.6 and kept in darkness on rotary shaker at 100 rpm at 28 $^{\circ}$C for 15, 30, 45 and 120 min. The two explants were placed on sterile tissue papers before transfer to co-cultivation medium (SIM containing 200 µM AS) and kept in the dark at 28 $^{\circ}$C for 3 days. After co-cultivation, the explants were washed with liquid SIM

containing 400 mg/L cefotaxime for 10 min to remove excess bacteria. Then explants were transferred to SIM supplemented with 200 mg/L cefotaxime and subcultured every 2 weeks for 1 month to eliminate bacteria . The inoculated explants were then transferred to selective medium (SIM containing 0.5 mM glyphosate) for early screening of transformed tissues. The cultures were maintained under 12.5 µmol/m^2/s illumination, 14 hours photoperiod at 26±2ºC.

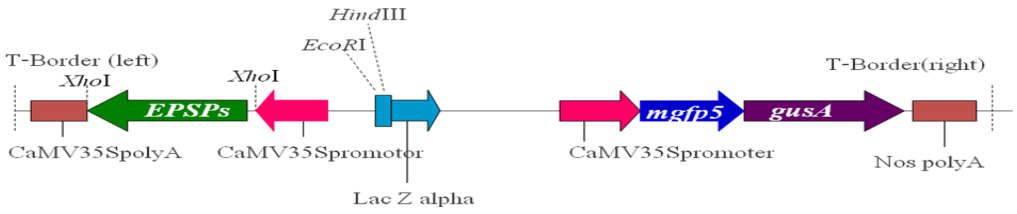

Figure 3.1 Schematic map of T-DNA region of the binary vector pCAMBIA1304-*EPSPs* containing the *gus* gene containing an intron as reporter genes and the *EPSPs* gene conferring glyphosate resistance.

Source: Rodpeawpan (2011)

Histochemical GUS assay and transient assessment

GUS assays were carried out using protocols described by Jefferson *et al.* (1987). GUS expression was observed by immersing inoculated explants in X-gluc buffer consisiting of 2 mM X-gluc, 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide. The explants were incubated overnight at 37°C in the dark, and washed with absolute methanol for overnight. The percentage of *gus* expression, in the presence of blue spots, were recorded and scored under stereomicroscope.

Molecular analysis of the transformed plantlets by PCR analysis and Dot blot hybridization

Genomic DNA was isolated from young leaf (0.05 g) of non-transformed and transformed plantlets after 1 months of culturing on selective medium by the CTAB (Hexadecyltrimethylammonium bromide) method (Doyle and Doyle, 1990). The *gus* gene fragment was amplified using forward primer sequence F-primer 5'-CTGCGACGCTCACACCGATAC-3' and reverse primer sequence R-primer 5'-TCACCGAAGTTCATGCCAGTCCAG-3'. The forward and reverse primer sequences for the *EPSPs* gene amplification were 5'-CCATTCCGCTCGAGATGGCACAAATTAACAACAT GGC-3' and 5'-ATCCACCGCTCGAGCGGTCATCAGGCAGCCTTCGTAT-3', respectively. The reaction mixture contained 1 µl of genomic DNA (20 ng), 0.5 µl of each primer (5 pmol), 4 µl of dNTP mix (1 mM each), 2 µl ml of PCR buffer, 0.1 µl of *Taq* DNA polymerase (1 U/ml) and the volume was adjusted to 20 µl with sterile distilled water. The PCR conditions included hot start at 96^{o}C for 2 m, followed by 30 cycles of denaturation (96^{o}C, 20 s), annealing (55^{o}C, 1 min) and extension (72^{o}C, 2 min), with a final extension of 5 min at 72^{o}C. PCR amplified products were resolved in 1.0 % agarose gel with ethidium bromide and visualized by gel documentation at 260 nm of UV.

For dot blot hybridization, 4 µg of the genomic DNA of non-transformed and transformed plantlets and 2 µL of PCR products were dropped on a nylon membrane (Hybond-N, Amersham). Blotted membrane was dried by incubation at 80 º C for 1 hour. The membrane was pre-hybridized in hybridization solution (5X SSC, 0.1 % N-lauroylsarcosine, 0.02 % SDS and 1X Blocking solution) for 1 hour at 65 ºC. Hybridization was performed with DIG-labeled DNA probe overnight at 65 º C, which was generated using the PCR DIG Probe Synthesis Kit (Roche Applied Science). Hybridized membrane was washed twice in low stringency buffer (2x SSC, 0.1 % SDS) for 15 min, twice in high stringency buffer (0.1x SSC, 0.1 % SDS) for 15 min and once in washing buffer (1x Maleic acid buffer, 0.3% Tween 20) for 10 min. The membrane was blocked in blocking solution

(Dilute 10x blocking solution 1: 10 with Maleic acid buffer) for 30 min. After that the anti-digoxigenin conjugate alkaline phosphate was added into blocking solution and incubated for 30 min. The membrane was then transferred to detection buffer (0.1 M Tris-HCl, 0.1 M NaCl) for 3 min at room temperature. Finally, the membrane was dropped by chemiluminescent substrate (CDP star™) and exposed to *Kodak BiomaX-Omat* film for autoradiography. The film was washed with developer and fixer solution after exposure in the cassette for 60 min.

Statistical analysis

Data were analyzed using CRD and the differences among means were separated using DMRT. All statistical analyses were performed at the level 5% using SAS (statistically analysis system).

Results and Discussion

Types of explants and inoculation time

Infection time is the most important step in the transformation process. However, conditions for inoculation may considerably affect the efficiency of the transformation processes. In this study, the transformation efficiency was compared under different inoculation times and types of explants (shoot tip and cotyledonary node). Between the two explants immersed in *A. tumefaciens* suspension at optical density of 600 nm at various times and co-cultivated for 3 days revealed that shoot tip explants gave the better results than nodal explants in all parameters tested. Transient GUS activity of shoot tip explants increased with inoculation time, reaching 72.11% GUS expression for 120 min, significant difference with the other time of inoculation (Table 3.1, Figure 3.2). The periods of inoculation seem to be effective for the efficient transfer of the T-DNA to plant cells (Kondo *et al.,* 2000). However, longer periods of inoculation affected survival rate of explants. The highest survival rate of shoots at 77.78% was obtained from 30 minute inoculation as shown

in Table 3.2 and Figure 3.3. Unfortunately, all nodal explants died after one month of culture on selection medium. Generally, inoculation time applied in transformation procedures varied from species to species. It took about 30 min for immature embryo of oil palm (Abdulah *et al.,* 2005) and for alfalfa calli (Zhang *et al.,* 2010), 40 min for embryogenic callus of *Parthenocissus tricuspidata* (Yang *et al.,* 2010), and up to 2 h for tobacco leaf ring (Vinad Kumar *et al.,* 2004). Interestingly, Blanc *et al.* (2006) reported that successful in transformation process of *H. brasiliensis* took only one second submerging integument calli in *A. tumefaciens* solution. Contrary results were obtained from the present study. Firstly, different explants were used. In the present study, shoot tip explants were applied. Organized tissues seem to resist to *A. tumefaciens* solution better than callus, thus, time required for inoculation is longer. Secondly, regenerability of those explants was far different. Callus was reported to be very sensitive to all stimulants applied *in vitro*, e.g. toxin, colchicine (Te-chato *et al*, 1995). Plantlet regeneration from callus just after treating with those chemicals was not reported. In case of nodal explants, they were died after 1 month of culture on selection medium, which was SIM medium containing 0.5 mM glyphosate due to the long inoculation period (120 min). This evident caused an overgrowth of *Agrobacterium* and decreased the survival rate (22.22%) of plant tissues. A similar result has also been reported in many plant species, such as *Parthenocissus tricuspidat* (Yang *et al.,* 2010) and alfalfa (Zhang *et al.,* 2010). A longer period of inoculation decreased the percentage of survival rate of explants co-cultured with *Agrobacterium*.

Table 3.1 Effect of inoculation times on percentage of GUS activities after 2 weeks of culture on selection medium.

Inoculation time	Percentage of GUS activities		Average[time]
	Shoot tip explants	Nodal explants	
15 min	28.89c	0.00d	14.44C
30 min	46.66b	0.00d	23.33BC
45 min	51.00b	0.00d	25.50B
120 min	72.11a	0.00d	36.05A
Average[explant]	49.66A	0.00B	**

** highly significant difference ($p \leq 0.01$)

Mean with different capital letter indicate significant difference among treatments and means with different small letter indicate significant difference among treatment combinations according to DMRT.

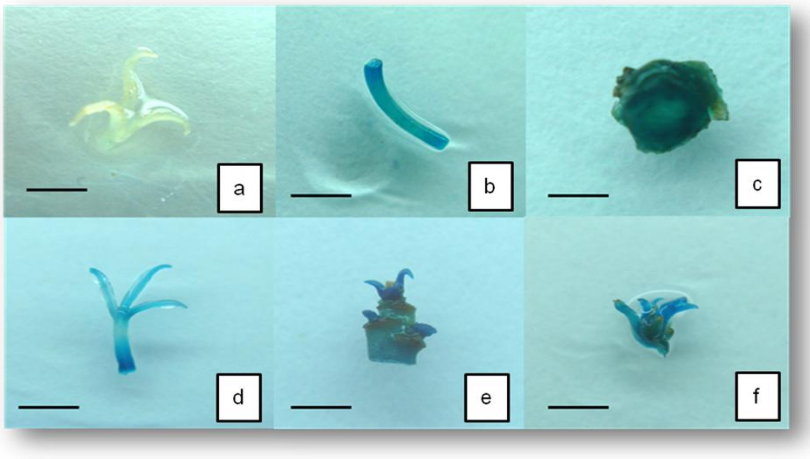

Figure 3.2 Histochemical assay of GUS activity in transgenic explants transformed by *Agrobacterium* harboring pCAMBIA1304-*EPSPs* containing the *gus* gene and the *EPSPs* gene conferring glyphosate resistance (bars= 5 mm).

(a) Leaf excised from non-transformed organ (control).

(b-f) Transformed petiole, stem, leaf, shoot and new forming shoot, respectively.

Table 3.2 Effect of inoculation time on percentage of glyphosate-resistant shoot tip and nodal explants of rubber tree after 4 weeks of culture on selection medium.

Inoculation time	Percentage of glyphosate-resistance explants		Average[time]
	Shoot tip explants	Nodal explants	
15 min	55.56ab	0.00d	27.78AB
30 min	77.78a	0.00d	38.89A
45 min	33.33bc	0.00d	16.67B
120 min	22.22cd	0.00d	11.11B
Average[source]	47.22A	0.00B	*

* significant difference ($p < 0.05$)

Mean with different capital letter indicate significant difference among treatments and means with different small letter indicate significant difference among treatment combinations according to DMRT.

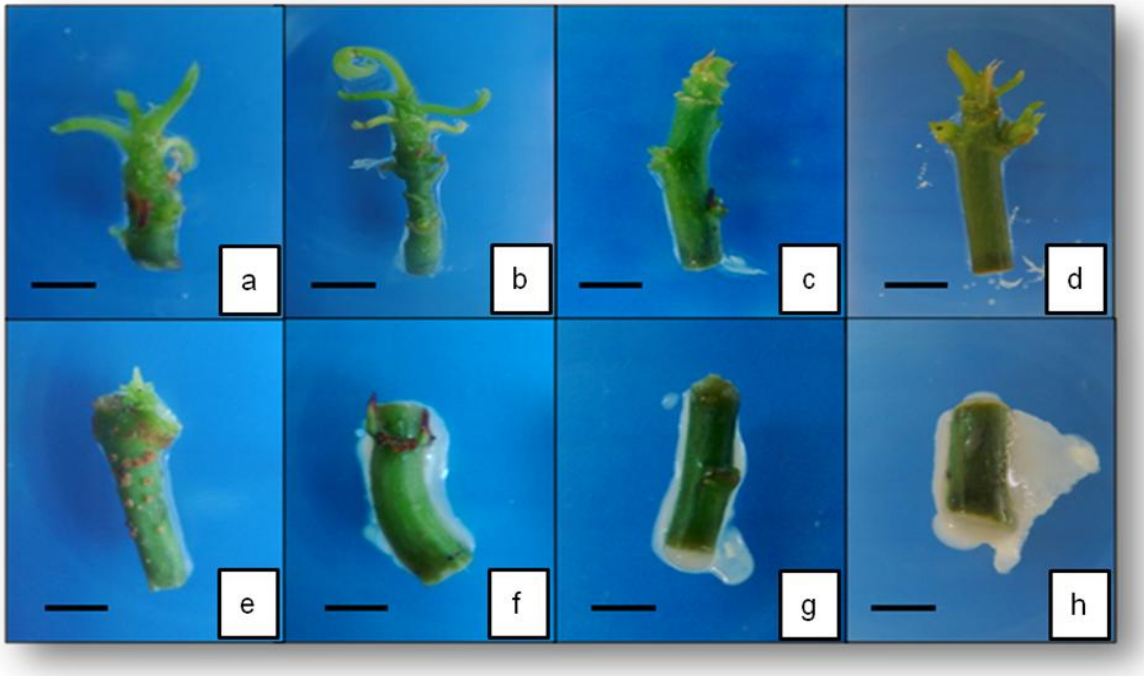

Figure 3.3 Morphological appearance of shoot tip and nodal explants inoculated with *Agrobacterium* harboring pCAMBIA subsequent to culture on co-cultivation medium for 3 days (bars = 5 mm).

(a-d) Shoot tips inoculated *A. tumefaciens* suspension at OD_{600} = 0.6 for 15, 30 45 and 120 min, respectively.

(e-h) Nodes inoculated *A. tumefaciens* suspension at OD_{600} = 0.6 for 15, 30 45 and 120 min, respectively.

Molecular analysis of the transformed plantlets by PCR analysis and dot blot hybridization

PCR analysis confirmed the presence of *gus* gene at size of 919 bps from transformed plantlets. The presence of *gus* genes was confirmed in 4 transgenic plants and in the plasmid DNA (Figure 3.4), whereas the corresponding band was not detected in the non-transgenic control. Dot blot hybridization confirmed the positive signals of *gus* gene in the genomic DNA of transformed plantlets of 4 samples. The positive transgenic plant samples developed dark black spots as well as the positive control sample indicating the success of gene transfer into plant genome (Figure 3.5). However, negative control showed slightly pale spots without a clear signal. This might be an error due to the procedure of washing the membrane with low concentration of low and high stringency buffers. These two buffers consisted of SSC solution (sodium chloride and sodium citrate) and SDS solution that might affect specific integration between *gus* probe and membrane.

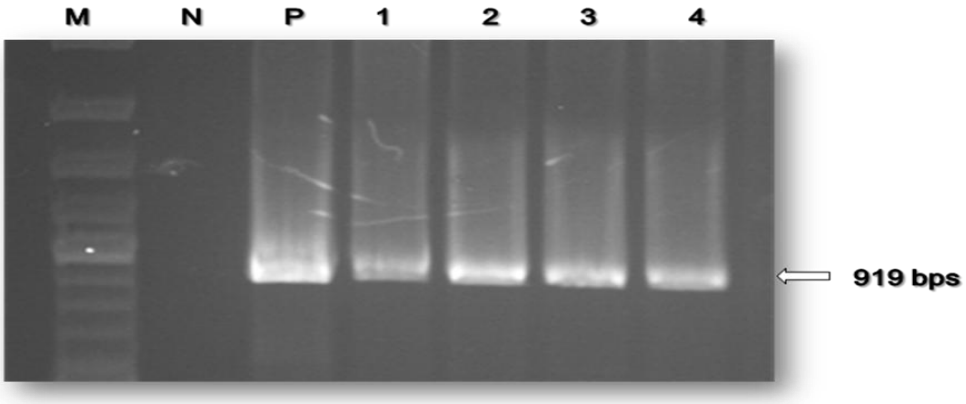

Figure 3.4 PCR analysis showed the presence of *gus* gene at 919 bps from different plant genomes.

M: marker at 100 bp

N: negative control of non-transformed genome

P: positive DNA control of *A. tumefaciens* plamid

1-4: shoot tips inoculated *A. tumefaciens* suspension at OD_{600} = 0.6 for 15, 30 45 and 120 min, respectively.

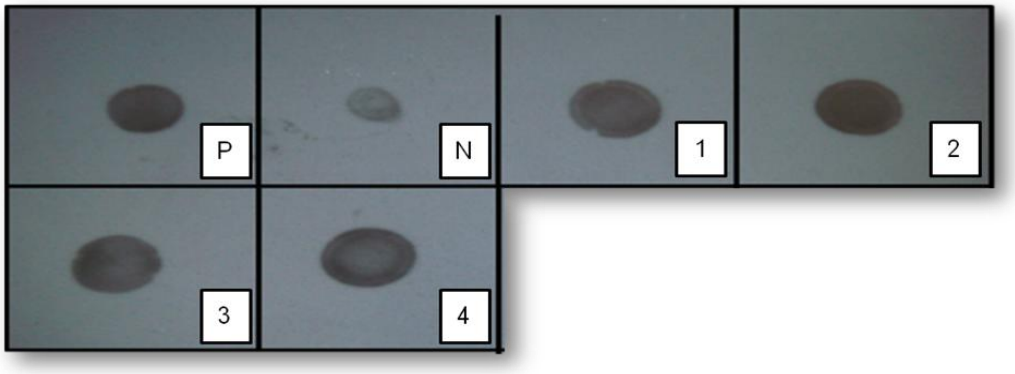

Figure 3.5 Dot blot hybridization using *gus* gene probe showed dark color dots into plant genome.

M: marker at 100 bp

N: negative control of non-transformed genome

P: positive DNA control of *A. tumefaciens* plamid

1-4: shoot tips inoculated *A. tumefaciens* suspension at OD_{600} = 0.6 for 15, 30 45 and 120 min, respectively.

CHAPTER III

Experiment II

The Effect of *Agrobacterium* Densities and Inoculation Times on Gene Transformation Efficiency in Rubber Tree

Introduction

Agrobacterium tumefaciens-mediated genetic transformation technique has most widely been used in many plant species due to its easy protocol without any special equipment. These techniques were developed on numerous *Hevea* clones, GL1, RRII 105 and PB 260 (Arokiaraj *et al.,* 1994; Jayashree *et al.,* 2003; Priya *et al.,* 2006; Montoro *et al.,* 2003; Lardet *et al.,* 2011). Montoro *et al.* (2003) developed genetic transformation protocol using *A. tumefaciens* in friable integument callus line (clone PB260) for a good frequency of integration of transgenic calli. An efficient genetic transformation procedure was investigated using highly integument-callus lines (Blanc *et al*., 2006) and GFP selection of transformants (Leclercq *et al.,* 2010). Nowadays, over-expression of endogenous genes involved in reactive oxygen species scavenging systems, such as MnSOD has been reported (Jayashree *et al.,* 2003). The target of transgene expression in latex cells was also attempted using the promoter from gene *HEV*2.1, which was the major latex Hevein protein (Montoro *et al.,* 2008). Moreover, genetic transformation protocol was developed using the transfer of a synthetic *EPSPs* transgene, as a conditional positive selectable marker, into commercially relevant zonal pelargoniums using an *A. tumefaciens* strain in combination with a novel step-down glyphosate selection system. Glyphosate is a commercial herbicide used in control of weed species which exerts its action on plants through inhibition of *EPSPs.* This chemical is not detoxified, and consequently here is no cross-protection afforded to evolve resistant weeds (Chen *et al.,* 2012). In addition, glyphosate is a highly mobile selection agent and translocates throughout the plant and so is less dependent on direct contact of the target tissue than some other selection agents (Howe *et al.,* 2002). However, transformation efficiency was depended on many factors, protocols and cultivar-dependent. Until now there is no report available regarding the gene transformation of glyphosate-resistant shoot of rubber tree. The introduction of foreign genes into plants was

assessed in the transformants by PCR analysis and Southern blot hybridizations. The process of genetic transformation in *H. brasiliensis* using *A. tumefacients* for glyphosate resistant rubber was optimized.

Materials and Methods

Plant material

Seeds from rubber tree clone Tjir1, naturally grown at Prince of Songkla University, Hat Yai campus, Songkhla province, Thailand, were collected and used as explants for zygotic embryo culture. After 2 weeks of culture, seedlings were obtained and they were excised into two parts, shoot tip and hypocotyl node. The two types of explants were cut into 1.5-2 cm in length and cultured on SIM supplemented with 5 mg/l BA, 3% sucrose and 0.05% activated charcoal as reported by Te-chato and Muangkaewngam (1992). The medium's pH was adjusted to 5.7 with 0.1 N HCl or KOH before adding 0.75% agar and autoclaved at 1.05 kg/cm^2, at 121 ºC for 15 min. The cultures were maintained at 28±0.5 ^{o}C under fluorescent lamps at light intensity of 12.5 µmol/m^2/s 14 hour photoperiod for 1 month. Single shoot at length of 1 cm was excised and used for transformation.

Bacterial plasmid

A. tumefaciens strain EHA105 containing the plasmid pCAMBIA1304-*EPEPs* which harbored *gus* and *EPSPs* genes (Figure 3.6) was used in this study. A single colony of this bacteria was pick out and suspended in 25 ml liquid LB medium (10 g/L tryptone, 5 g/L Bacto yeast extract, 5 g/L NaCl, pH 7.0) containing 50 mg/L kanamycin and incubated on a rotary shaker at 100-150 rpm in the dark at 28ºC. After proliferation in LB medium overnight the cells were collected and resuspended in SIM. The suspension of *A.*

tumefaciens was adjusted by spectrophotometer at OD of 600 nm and used for transformation.

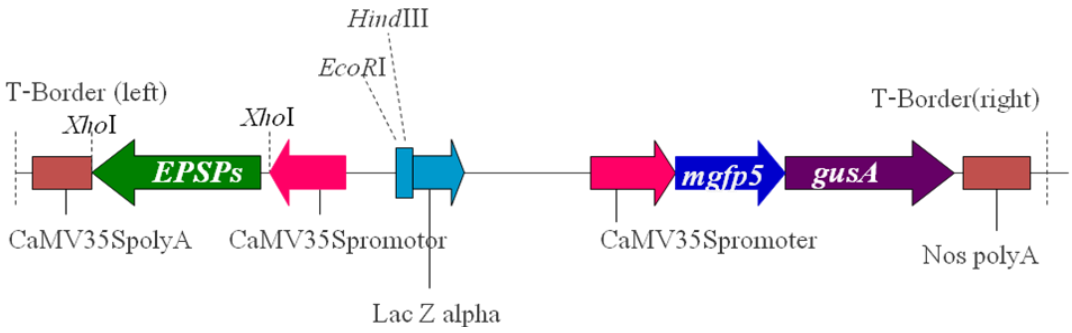

Figure 3.6 Schematic map of T-DNA region of the binary vector pCAMBIA1304-*EPSPs* containing the *gus* gene containing an intron as reporter genes and the *EPSPs* gene conferring glyphosate resistance.

Source: Rodpeawpan (2011)

***Agrobacterium* densities and inoculation time**

The shoot tips were immersed in 25 ml of the *A. tumefaciens* suspension at optical density (OD_{600}) at 0.3, 0.6 and 0.9. The cultures were maintained on rotary shaker in darkness at 100 rpm at 28°C for 15, 30 and 60 min. The explants were placed on sterile tissue papers before transfer to co-cultivation medium which was SIM containing 200 µM acetosyringone. The co-cultivation was kept in the dark at 28°C for 3 days. After co-cultivation, the explants were washed with liquid SIM containing 400 mg/L cefotaxime for 10 min to remove excess bacteria. Then explants were transferred to SIM supplemented with 200 mg/L cefotaxime to eliminate bacteria for 2 weeks. The inoculated explants were then transferred to selective medium (SIM containing 0.5 mM glyphosate). After 2 weeks of culture, inoculated explants were cultured on SIM containing 2 mM glyphosate for early

screening of transformed tissues and subcultured every 2 weeks. The cultures were maintained under 12.5 µmol/m^2/s illumination, 14 h photoperiod at 26±2ºC.

Histochemical GUS assay and selection of putative transformants

GUS assays were carried out using protocols described by Jefferson *et al*. (1987). GUS expression was observed by immersing inoculated explants in X-gluc buffer consisting of 2 mM X-gluc, 100 mM sodium phosphate buffer (pH 7).0, 0.5 mM potassium ferricyanide, and 0.5 mM potassium ferrocyanide. The explants were incubated overnight at 37^{o}C in the dark, and washed with absolute methanol for overnight. The percentage of *gus* expression which was the blue percentage per explant, was recorded and scored under stereomicroscope.

For selection, glyphosate was used for selection of putative transformants. Non-inoculated and inoculated shoots were cultured on selective medium (SIM supplemented with 2 mM glyphosate). After 1 and half months of inoculation, the percentage of glyphosate-resistant shoots [% resistant = (number of survival shoots / total number of shoots) x 100] was recorded.

Molecular analysis of the transformed plantlets by PCR analysis, dot blot hybridization and Southern blot PCR hybridization

Genomic DNA was isolated from young leaf (50 mg) of non-transformed and transformed plantlets after 1 and half months of culturing on selective medium by the CTAB method (Doyle and Doyle, 1990). The *gus* gene fragment was amplified using forward primer sequence F-primer 5'-CTGCGACGCTCACACCGATAC-3' and reverse primer sequence R-primer 5'-TCACCGAAGTTCATGCCAGTCCAG-3'. The forward and reverse primer sequences. For the *EPSPs* gene amplification 5'-CCATTCCGCTCGAGATGGCA CAAATTAACAACATGGC-3' and 5'-ATCCACCGCTCGAGCGGTCATCAGGCAGCCTTC

GTAT-3', respectively were applied. The reaction mixture contained 1 µl of genomic DNA (20 ng), 0.5 µl of each primer (5 pmol), 4 µl of dNTP mix (1 mM each), 2 µl ml of PCR buffer, 0.1 µl of *Taq* DNA polymerase (1 U/ml) was mixed together and adjusted to 20 µl with sterile distilled water. The PCR reaction started at 96°C for 2 min, followed by 30 cycles of denaturation (96°C, 20 sec), annealing (55°C, 1 min) and extension (72°C, 2 min), with a final extension of 5 min at 72°C. PCR amplified products were separated in 1.0 % agarose gel with ethidium bromide and visualized by gel documentation at 260 nm of UV.

For dot blot hybridization, 4 µg of the genomic DNA of non-transformed and transformed plantlets and 2 µL of PCR products were dropped on a nylon membrane (hybond-N, Amersham). Blotted membranes were dried by incubation at 80ºC for 1 h. The membranes were pre-hybridized in hybridization solution (5X SSC, 0.1 % N-lauroylsarcosine, 0.02 % SDS and 1X blocking solution) for 1 h at 65 ºC. Hybridization was performed with DIG-labeled DNA probe (*gus* or *EPSPs* gene) overnight at 65ºC, which was generated using the PCR DIG Probe Synthesis Kit (Roche Applied Science). Hybridized membrane was washed twice in low stringency buffer (2X SSC, 0.1% SDS) for 15 min, twice in high stringency buffer (0.1X SSC, 0.1% SDS) for 15 min and once in washing buffer (1X maleic acid buffer, 0.3% tween 20) for 10 min. The membrane was blocked in blocking solution (dilute 10X blocking solution 1:10 with maleic acid buffer) for 30 min. After that, the anti-digoxigenin conjugate alkaline phosphate was added into blocking solution and incubated for 30 min. The membrane was then transferred to detection buffer (0.1 M Tris-HCl, 0.1 M NaCl) for 3 min at room temperature. Finally, the membrane was dropped by chemiluminescent substrate (CDP star™) and exposed to Kodak BiomaX-Omat film for autoradiography. The film was washed with developer and fixer solution after exposure in the cassette for 60 min.

For Southern blot PCR hybridization, 15 µL of PCR products (1-2 mg/µL) were separated by 1 % agarose gel electrophoresis. The gel was treated with 0.25 N HCl to

depurinate briefly the DNA and then denatured with an alkaline solution for 30 min and neutralized for 30 min. The denatured DNA was then transferred to a nylon membrane (hybond-N, Amersham). Blotted membrane was dried by incubation at 80 ºC for 1 h. The blotted membrane was hybridized and detected using the same protocol according to dot blot hybridization as describe above.

Statistical analysis

Data were subjected to ANOVA analysis and significant difference was determined with the balance analysis test with a level of significance at $p \leq 0.05$ using SAS. A 3x3 factorial in completely randomized design with three replicates was used. Each replication consisted of 9 samples.

Results and Discussion

Agrobacterium densities and inoculation times

The explants immersed in *A. tumefaciens* suspension at optical density of 600 nm at three different concentrations (0.3, 0.6 and 0.9) and three inoculation times (15, 30 and 60 min) revealed that shoot explants immersed in *A. tumefaciens* suspension at density of 0.9 gave the highest GUS expression in all parameters tested. In addition, transient GUS activity increased with increase in inoculation time, reaching 57.67%. GUS expression at inoculation period of 60 min gave the highest result, significant difference with the other times of inoculation (Table 3.3, Figure 3.7). In addition, *A. tumefaciens* density played significantly different result in gene transformation. Optical density at 600 nm of 0.9 gave the highest GUS expression (53.22%). There was no interaction effect between inoculation times and *A. tumefaciens* densities. Nevertheless, the concentration of *A. tumefaciens* affected survival rate of explants (Figure 3.8). The result showed that putative transformants shoots died after 1 and half month of culture on selection medium,

which was SIM medium containing 0.5-2 mM glyphosate due to high density of *A. tumefaciens* cell (OD_{600}=0.9). This evident caused by an overgrowth of *A. tumefaciens* and decreased in the survival rate of plant tissues. For this reason, the putative transformants with the highest glyphosate-resistant shoots were obtained from optical density at 0.6. A similar result has also been reported in many plant species, such as alfalfa (Zhang *et al.,* 2010) and *Parthenocissus tricuspidat* (Yang *et al.,* 2010). However, inoculation time was not significantly different in percentage of glyphosate-resistant shoots. Significant interaction was not found between inoculation time and *A. tumefaciens* density in glyphosate-resistant shoots.

In case of inoculation time, the highest result of transient GUS activity was obtained from 60 min inoculation. It was clearly shown that shoot explants immersed in *A. tumefaciens* suspension for 60 min gave the highest GUS expression in all treatments tested. However, longer period of inoculation decreased the percentage of survival rate of explants co-cultured with *A. tumefaciens*. According to this result, the inoculation period was critical factor for gene transformation. The highest survival rate of shoots was obtained from 30 min inoculation (Table 3.4 and Figure 3.9). Indeed, the inoculation time of *Agrobacterium* has a close relation with penetration or transmission of T-DNA in the plant tissue. The presence of a larger number of bacterial cells might enhance both the number of transformation events and tissue response related to biotic stress. Kondo *et al.* (2000) reported that the periods of inoculation seem to be effective for transfer of the T-DNA into plant cells, and longer periods of inoculation gave negative effect on survival rate of explants. Normally, the inoculation time applied in transformation procedures is about 30 min for immature embryo of oil palm (Abdulah *et al.,* 2005) and alfalfa calli (Zhang *et al.,* 2010), 40 min for embryogenic callus of *Parthenocissus tricuspidata* (Yang *et al.,* 2010), and up to 2 h for tobacco leaf ring (Vinod *et al.,* 2004). Interestingly, Blanc *et al.* (2006) reported that successful in transformation process of rubber tree took only one second

submerging calli in *A. tumefaciens* suspension. Contrary results were obtained in the present study. Firstly, different explants type was used. In the present study, shoot explants were applied. Organized tissues seem to resist to *A. tumefaciens* solution better than callus, thus time required for inoculation might be longer. Secondly, regenerability of those explants was far different. Callus of rubber was reported to be very sensitive to all stimulants applied *in vitro*, e.g. toxin, colchicine (Te-chato *et al.,* 1995). Plantlet regeneration from callus just after treating with those chemicals was not reported. Therefore, *A. tumefaciens* desity at optical density 600 nm of 0.6 and inoculation time for 30 min could improve transient GUS expression at 46.67% (Table 3.3) and glyphosate-resistant shoot 48.67% (Table 3.4) for gene transformation procedure in rubber tree.

Molecular analysis of the transformed plantlets by PCR, dot blot hybridization and Southern blot PCR hybridization

To prove the presence of the *gus* and *EPSPs* gene in transformed rubber tree, PCR analysis was conducted to evaluate putative transformants, along with non-transgenic plant (negative control). Specific primers for the *gus* gene were designed to amplify a 919 bps DNA fragment. The presence of *gus* genes was confirmed in nine transgenic plants and in the plasmid DNA, whereas the corresponding band was not detected in the non-transgenic control (Figure 3.10a). For *EPSPs* gene, 7 transformed plantlets out of 9 plantlets (77.78%) showed the positive results of that gene at 1,600 bps, but sample in lane number 9 showed slightly pale band (Figure 3.10b).

In case of dot blot hybridization using *gus* gene probe, the genomic DNA of nine samples showed dark color dots indicating the success of gene transfer into plant genome (Figure 3.11a). On the other hand, dot blot hybridization using *EPSPs* gene showed the positive results of 7 samples from 9 samples (77.78%). The positive transgenic plant

samples developed dark black spots as well as the positive control sample, while the non-transformed plantlet samples did not show the dark spots (Figure 3.11b).

For Southern PCR hybridization it was clearly confirmed the presence of *gus* gene and *EPSPs* gene at size of 919 bps and 1,600 bps, respectively in plant genome. The bands of DNA from non-transformed and transformed shoot (OD_{600}=0.9, 60 min) in lane number 9 didn't appear (Figure 3.12c). Moreover, Southern PCR hybridization using *EPSPs* gene gave the same result in dot blot hybridization. Only seven positive transgenic plant samples developed dark black bands as well as the positive control samples, while the non-transformed samples did not show the dark bands (Figure 3.12d). The reason might be due to incompleteness of the transportation of T-DNA from *Agrobacterium* to plant genome. Enzyme endonuclease cut T-DNA at RB (right border) from Ti plasmid and inserted RB border into plant genome before LB (left border). Right border connected with *gus* gene while *EPSPs* gene locates far away (Figure 3.1). So it is possible that incomplete transfer of T-DNA was performed. For this result only *gus* gene (reporter gene) at the first part was sent to plant genomes while the *EPSPs* genes were not. Thus, in the present study, all of transgenic samples were presented of *gus* gene, but not all for *EPSPs* gene.

Table 3.3 Effect of *A. tumefaciens* densities and inoculation times on transient expression of the *gus* gene (%) in rubber tree after 4 weeks of transformation.

A. tumefaciens densities	Transient expression of the *gus* gene (%)			
	Inoculation times			
	15 min	30 min	60 min	Average [density]
0.3	35.33c	37.67bc	38.33bc	37.11B
0.6	40.47abc	46.67abc	54.33ab	47.22A
0.9	48.33abc	53.67ab	57.67a	53.22A
Average [time]	41.44	46.00	50.11	
C.V. (%)	20.51			
F (Rep.)	ns			
F (Density)	*			
F (Time)	ns			
F (Density x Time)	ns			

ns not significant difference, * significant difference ($p \leq 0.05$))

Means with different small letter indicate significant difference among treatments and mean with different capital letters indicate significant differences among treatment combination. The data are the means from nine samples with three replicates.

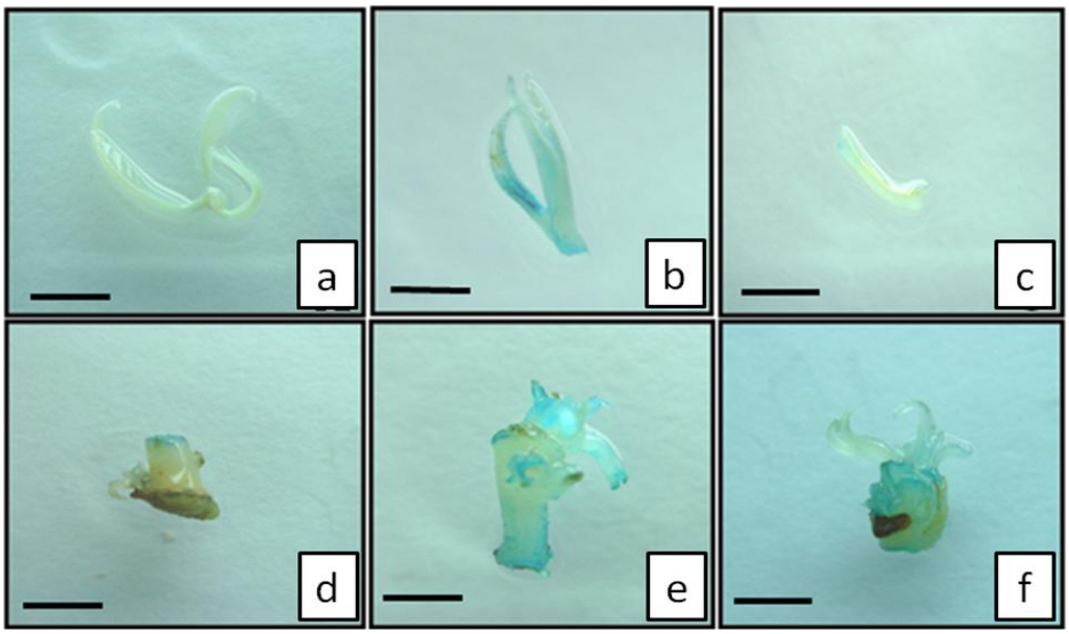

Figure 3.7 Histochemical assay of GUS activity in transgenic explants transformed by *Agrobacterium* harboring pCAMBIA1304-EPSPs containing the *gus* gene and the *EPSPs* gene conferring glyphosate resistance (bars= 5 mm).

(a) non-transformed leaf

(b) transformed leaf

(c) transformed petiole

(d) transformed stem

(e) transformed shoot

(f) transformed new forming shoot

Table 3.4 Effect of *A. tumefaciens* densities and inoculation times on glyphosate resistance shoots (%) of rubber tree after 1 and half months of transformation.

A. tumefaciens densities	Glyphosate resistance shoots (%)			
	Inoculation times			
	15 min	30 min	60 min	Mean density
0.3	33.33bc	36.33abc	35.67acb	35.11B
0.6	48.33a	48.67a	45.67ab	37.56A
0.9	28.33c	25.67c	26.67c	26.89C
Mean time	36.89	36.67	36.00	
C.V. (%)	18.99			
F (Rep.)	ns			
F (Density)	*			
F (Time)	ns			
F (Density x Time)	ns			

ns not significant difference * significant difference ($p \leq 0.05$)

Means with different small letters indicate significant difference among treatments and mean with different capital letters indicate significant difference among treatment combinations.

The data are the means from nine samples with three replicates.

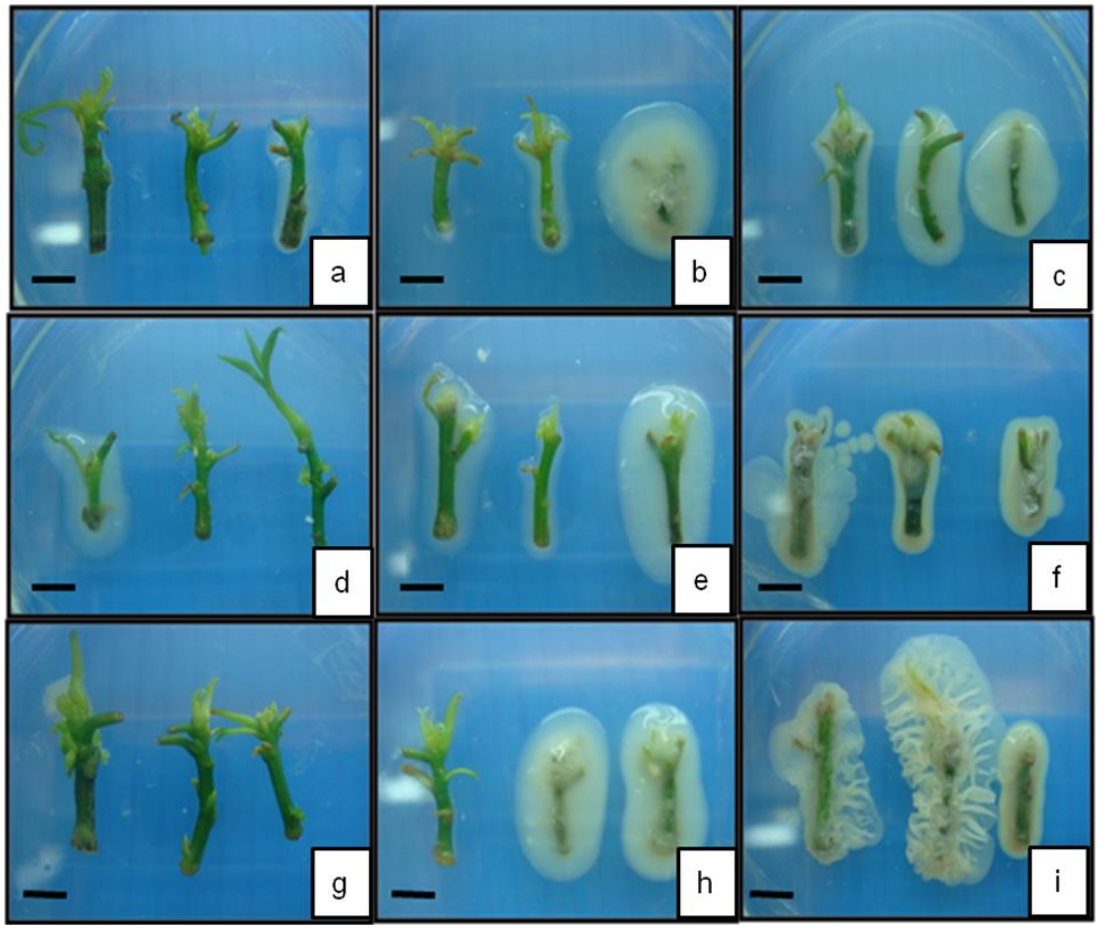

Figure 3.8 Morphological appearance of shoot explants inoculated with *Agrobacterium* harboring pCAMBIA1304-*EPSPs* at various *A. tumefaciens* densities and inoculation times subsequent to culture on co-cultivation medium for 3 days (bars = 5 mm).

(a-c) Inoculation at OD_{600} = 0.3 for 15, 30 and 60 min, respectively

(d-f) Inoculation at OD_{600} = 0.6 for 15, 30 and 60 min, respectively

(g-i) Inoculation at OD_{600} = 0.9 for 15, 30 and 60 min, respectively

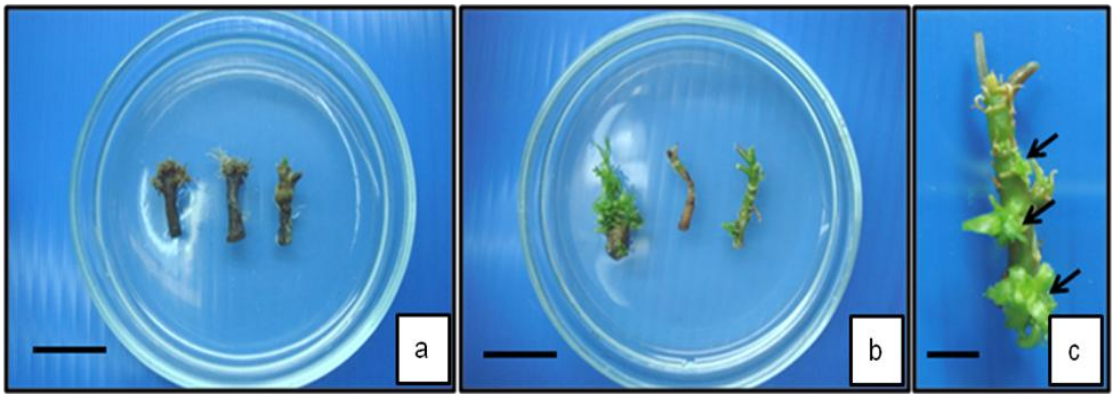

Figure 3.9 Growth of shoots cultured on glyphosate containing SIM after culture for one and half months (bar = 1 cm).

(a) control (non-transformed shoot)

(b) shoot inoculated *A. tumefaciens* with OD_{600} = 0.6 for 30 min

(c) new-formation of shoots from axillary buds after culture on selection medium for one and half month.

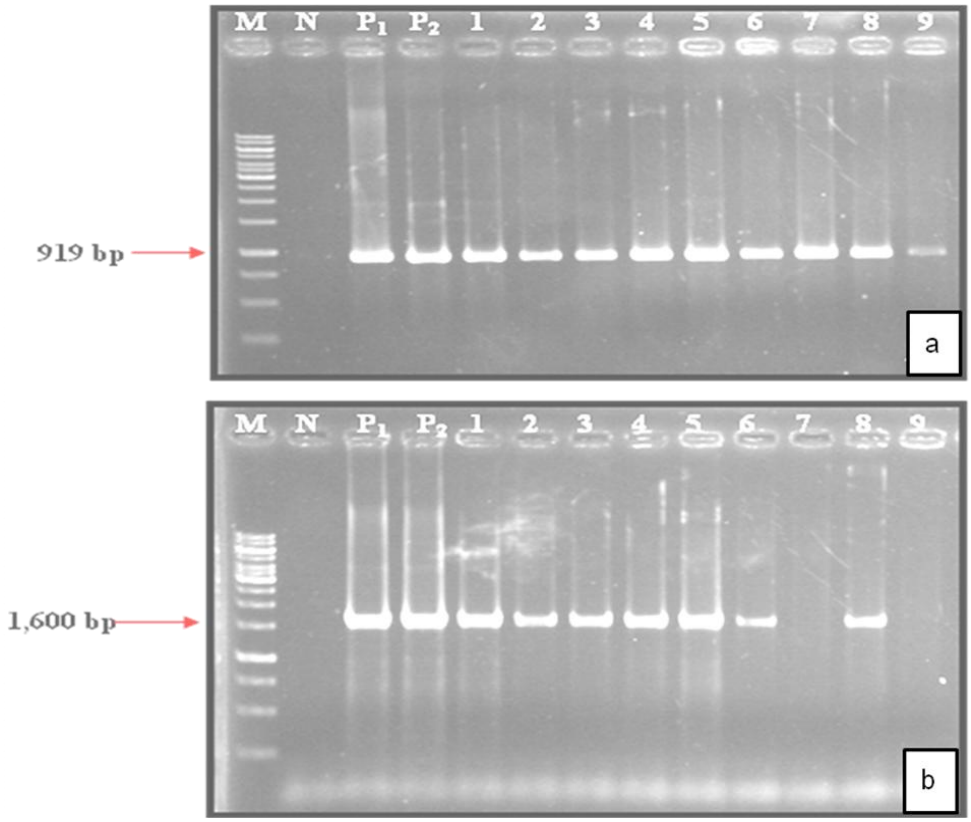

Figure 3.10 PCR analysis of DNA extracted from leaf of shoots transformed by *A. tumefaciens* containing pCAMBIA1304-*EPSPs* showed the presence of *gus* gene at 919 bps(a) and *EPSPs* gene at 1,600 bps(b).

M: marker

N: negative control

P: positive DNA control

1-3: shoot tips inoculated *A. tumefaciens* at OD_{600}=0.3 for 15, 30 and 60 min, respectively.

4-6: shoot tips inoculated *A. tumefaciens* at OD_{600}=0.3 for 15, 30 and 60 min, respectively.

7-9: shoot tips inoculated *A. tumefaciens* at OD_{600}=0.3 for 15, 30 and 60 min, respectively.

Figure 3.11 Detection of (a) *gus* gene and (b) *EPSPs* gene in genomic DNA of transformed shoots after one and half months on selection medium by dot blot hybridization.

M: marker

N: negative control

P: positive DNA control

1-3: shoot tips inoculated *A. tumefaciens* at OD_{600}=0.3 for 15, 30 and 60 min, respectively.

4-6: shoot tips inoculated *A. tumefaciens* at OD_{600}=0.3 for 15, 30 and 60 min, respectively.

7-9: shoot tips inoculated *A. tumefaciens* at OD_{600}=0.3 for 15, 30 and 60 min, respectively.

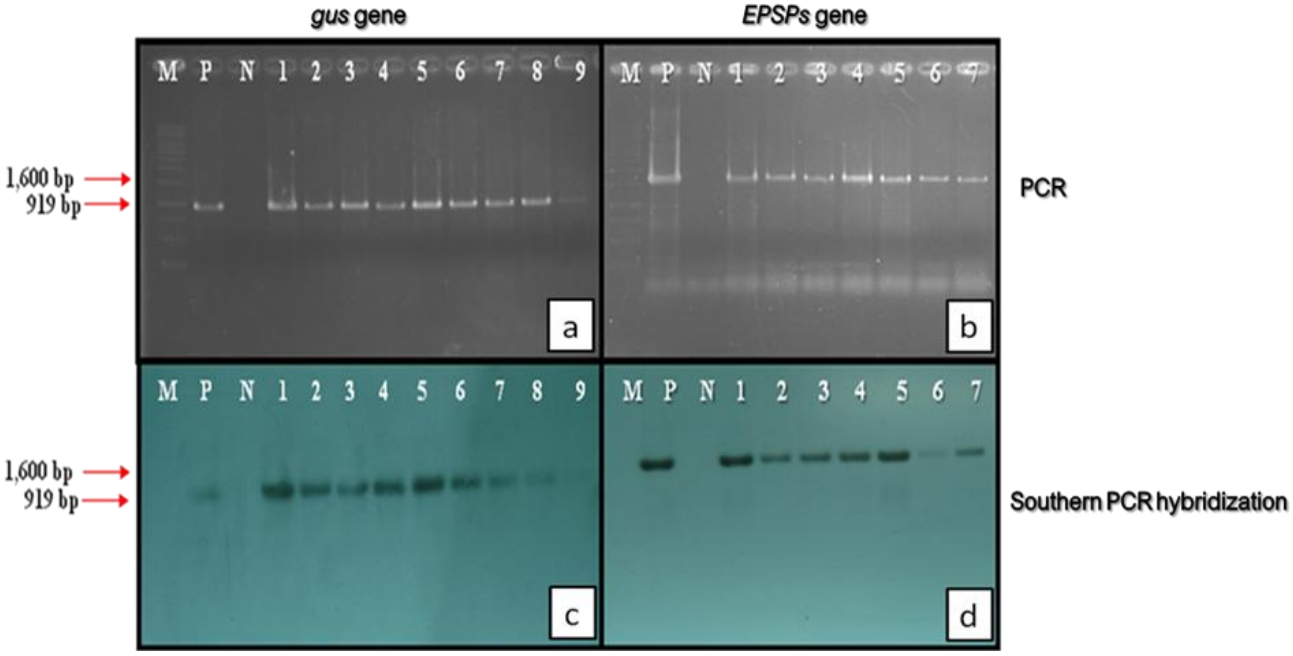

Figure 3.12 Detection of *gus* gene (a, c) and *EPSPs* gene (b, d) in transformed shoot after one and half months on selection medium by PCR (a, b) and Southern PCR hybridization (c, d).

M: marker

N: negative control

P: positive DNA control

1-3: shoot tips inoculated *A. tumefaciens* at OD_{600}=0.3 for 15, 30 and 60 min, respectively.

4-6: shoot tips inoculated *A. tumefaciens* at OD_{600}=0.3 for 15, 30 and 60 min, respectively.

7-9: shoot tips inoculated *A. tumefaciens* at OD_{600}=0.3 for 15, 30 and 60 min, respectively.

CHAPTER V

Concluding Remarks

Concluding Remarks

Mature zygotic embryos with partly endosperm cultured on MS medium supplemented with 10 mg/L BA and 1 mg/LIAA under light condition for 13 days gave the highest percentage of germination at 93.3. For multiple shoot induction, shoot tip explants cultured on 5 mg/L BA and 1 mg/L IBA containing medium gave the best result in number of shoots at 4.67 shoots per explant after 40 days of culture. For shoot elongation, the result showed that placing stem explants horizontally on surface of shoot induction medium without activated charcoal gave the best result in shoot numbers at 2.3 shoots per explant and leaf number at 5.33 leaves per shoot.

For assessment of transformation efficiency, *A. tmefaciens* containing pCAMBIA carrying agronomical trait for glyphosate-resistant *in vitro* was conducted. The bacteria carrying plasmid pCAMBIA 1304, harboring *gus* gene as screenable marker genes and *EPSPs* gene was used. The shoot tips were immersed in *A. tumefaciens* suspension at optical densities (OD_{600}) nm at 0.6 for 30 min gave the higher survival rate (48.67%) after being cultured on glyphosate containing MS medium for one and half months. Assessment of transformed shoots revealed positive results in GUS histochemical assay. The presence of the *gus* and *EPSPs* genes in transformed rubber tree were confirmed by PCR technique. The presence of *gus* genes was confirmed at 919 bps in nine transgenic plants (100%), whereas the *EPSPs* gene showed the positive results at 1,600 bps of 7 samples from 9 samples (77.78%). In case of dot blot hybridization using *gus* gene probe, the genomic DNA of nine samples showed dark color dots indicating the success of gene transfer into plant genome (Figure 3.11a). Similar result obtained from dot blot hybridization using *EPSPs* gene was showed the positive results of 77.78%. For Southern PCR hybridization it was clearly confirmed the presence of *gus* gene (9 samples) and *EPSPs* gene (7 samples) at size of 919 bps and 1,600 bps, respectively.

Future prospect

These results suggested that the improved medium was optimized microcutting conditions. The advantage of this study is *in vitro* micrografting in further experiment. Not only microcutting techniques have great potential for *in vitro* micropropagation and conservation of genetic resources of rubber tree, but also embryogenic callus or somatic embryogenesis is good source of plant material for gene transformation. Gene transformation of rubber with desired gene is very important for the development of cultivars. Finally, genetic engineering is certainly a more hopeful method for a high capability of plant improvement when specific genetic changes need to be made in a short time.

REFERENCES

Abdullah, R., A. Zainal, W.Y. Heng, L.C. Li, Y.C. Beng, L.M. Phing, S.A. Sirajuddin, W.Y.S. Ping, J.L. Joseph, S.A. Jusoh, M.R. Muad and Y.L. Huey. 2005. Immature embryo: A useful tool for oil palm (*Elaeis guineensis* Jacq.) genetic transformation studies. Journal Biotechnology 8: 24-34.

Arokiaraj, P., H. Jones, K.F. Cheong, S. Coomber and B.V. Charlwood. 1994. Gene insertion into *Hevea brasiliensis*. Plant Cell Reports 13: 425-431.

Arokiaraj, P., F. Rueker, E. Obermayr, A.R. Shamsul Bahri, J. Hafsah, D.C. Carter and H.Y. Yeang. 2002. Expression of human serum albumin in transgenic *Hevea brasiliensis*. Journal of Rubber Research 5: 157-166.

Asokan, M.P., K. Narasimhan, P. K. Jayasree, A. Thulaseedharan, S.S. Kumari, R.G. Kala and R. Jayashree. 2001. Isozyme markers for distinguishing embryogenic callus from non embryogenic during somatic embryogenesis in *Hevea brasiliensis* Muell. Arg. Journal of Tree Science 20: 78-85.

Bouychou, J. G. 1953. La culture *in vitro* des tissues d' *Hevea*. Archives of Rubber Cultivation 30: 50-53.

Boase, M.R., R.W. Harriman, F.D. Smith and S.C. Deroles. 2012. Herbicide-resistant, transgenic zonal pelargoniums produced by step-down glyphosate selection and plantlet recovery in the present of aromatic amino acids. In Vitro Cellular and Developmental Biology Plant 48: 313-323

Belarmino, M.M. and M. Mii. 2000. *Agrobacterium*-mediated genetic transformation of phalaenopsis orchid. Plant Cell Reports 19:435-442.

Blanc, G., C. Baptiste, G. Oliver, F. Martin and P. Montoro. 2006. Efficient *Agrobacterium tumefaciens*-mediated transformation of embryogenic calli and regeneration of *Hevea brasiliensis* Mull Arg. plants. Plant Cell Reports 24: 724-733.

Carron, M.P. and F. Enjalric. 1982. Studies on vegetative propagation of *Hevea brasiliensis* by somatic embryogenesis and *in vitro* microcutting. Tokyo: In Fujiwara.

Chen, S., S. Peng, G. Huang, K. Wu, X. Fu and Z. Chen. 2003. Association of decreased expression of a Myb transcription factor with the TPD (tapping panel dryness) syndrome in *Hevea brasiliensis*. Plant Molecular Biology 51: 51–58.

Chen, S., M. Chai, Y. Jia, Z. Gao, L. Zhang, M. Gu, W. Lin and L. Wang. 2012. *In vitro* selection of glyphosate-tolerant variants from long-term callus cultures of *Zoysia matrella* [L.] Merr. Plant Cell, Tissue and Organ Culture 105: 157-166.

Cheng, M., J.E. Fry, S. Pang, H. Zhou, C. Hironaka, D.R. Duncan, T.W. Conner and Y. Wan. 1997. Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiology 115: 971-980.

Cle´ment-Demange, A., P.M. Priyadarshan, T.T.H. Tran and P. Venkatachalam. 2007. *Hevea* rubber breeding and genetics. Plant Breeding 4(29): 177-283.

Doyle, J.J. and J.L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Enjalric, F. and M.P. Carron. 1982. Microbouturagew *in vitro* de jeunes plants d' *Hevea brasiliensis*. French Academy of Sciences 295: 259-264.

Fan, Q., X.P. Xu, X.L. Huang and B.J. Li. 2002. Callus formation and plant regeneration of indica rice variety Pei'ai 64S. Acta Botanica Boreali-Occidentalia Sinica 22: 1469-1473.

Hagel, J.M., E.C. Yeung, P.J. Facchini and M. Got. 2008. The secret life of laticifers. Plant Science 13: 631-639.

Howe, A.R., C.S. Gasser, S.M. Brown, S.R. Padgette, J. Hart, G.B. Parker, M.E. Fromm and C.L. Armstrong. 2002. Glyphosate as a selective agent for the production of fertile transgenic maize (*Zea mays* L.) plants. Molecular Breeding 10: 153–164.

Hu, T., S. Meltz, C. Chay, H.P. Zhou, N. Biest, G. Chen, M. Cheng, X. Feng, M. Radionenka, F. Lu and J.E. Fry. 2003. *Agrobacterium*-mediated large scale transformation of wheat (*Triticum aestivum* L.). Plant Cell Reports 21: 1010-1019.

Hua, Y.W., T.D. Huang and H.S. Huang. 2010. Micropropagation of self-rooting juvenile clones by secondary somatic embrogenesis in *Hevea brasiliensis*. Plant Breeding 129: 202-207.

Jayashree, P.K., M.P. Asokan, S. Sobha, L.S. Ammal, K. Rekha, R.G. Kala, R. Jayasree and A. Thulaseedharan. 1999. Somatic embryogenesis and plant regeneration from immature anthers of *Hevea brasiliensis* Mull. Arg. Current Science 76: 1242-1245.

Jayashree, R., K. Rekha, P. Venkatachalam, S.L. Uratsu, A.M. Dandekar, P. Kumari Jayasree,R.G. Kala, P. Priya, S.S. Kumari, S. Sobha, M.P. Ashokan, M.R. Sethuraj and A. Thulaseedharan. 2003. Genetic transformation and regeneration of rubber tree (*Hevea brasiliensis* Muell. Arg) transgenic plants with a constitutive version of an anti-oxidative stress superoxide dismutase gene. Plant Cell Reports 22: 201–209.

Jayasree, P.K. and A. Thulaseedharan. 2001. Gibberellic acid regulated embryo induction and germination in *Hevea brasiliensis*. Indian Journal of Natural Rubber Research 14: 106-111.

Jefferon, R.A., T.A. Kananagh and M.W. Bevan. 1987. GUS fusion: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plant. EMBO Journal 6: 3301-3306.

Kondo T., H. Hasegawa and M. Suzuki. 2000. Transformation and regeneration of garlic (*Allium sativum* L.) by A*grobacterium* mediated gene transfer. Plant Cell Reports 19: 989-993.

Kouassi, K.M., K.E. Koffi, Y.M. Gnagne, O. N'nan, Y. Coulibaly and A. Sangare. 2008. Production of *Hevea brasiliensis* embryos from *in vitro* culture of unpollinated ovules. Biotechnology 7: 793-797.

Lardet, L., F. Martin, F. Dessailly, M.P. Carron and P. Montoro. 2007. Effect of exogenous calcium on post-thaw growth recovery and subsequent plant regeneration of cryopreserved embryogenic calli of *Hevea brasiliensis* Muell. Arg. Plant Cell Reports 26: 559-569.

Lardet, L., J. Leclercq, E. Benistan, F. Dessailly, G. Oliver, F. Martin and P. Montoro. 2011. Variation in GUS activity in vegetatively propagated *Hevea brasiliensis* transgenic plants. Plant Cell Reports 30: 1847-1856.

Le, V.Q., J. Belles-Isles, M. Dusabenyagusani and F.M. Tremblay. 2001. An improved procedure for production of white pruce (*Picea glauca*) transgenic plants using *Agrobacterium tumefaciens*. Journal of Experimental Botany 52: 2089-2095.

Leclercq, J., L. Lardert, F. Martin, T. Chapuset, G. Oliver and P. Montoro. 2010. The green flurescent protein as an efficient selection marker for *Agrobacterium tumefaciens*-mediated transformation in *Hevea brasiliensis* (Muell. Arg.). Plant Cell Reports 29: 513-522.

Lespinasse, D., L. Grivet, V. Troispoux, M. Rodier-Goud, F. Pinard and M. Seguin. 2000. Identification of QTLs involved in the resistance to South American leaf blight (*Microcyclus ulei*) in the rubber tree. Theoretical and Applied Genetics 100: 975-984.

Montoro, P., W. Rattana, V.P. Renaud, N.M. Ferri, Y. Monkolsook, R. Kanthapura and S. Adunsadthapong. 2003. Production of *Hevea brasiliensis* transgenic embryogenic callus lines by *Agrobacterium tumefaciens*: roles of calcium. Plant Cell Reports 21: 1095-1102.

Montoro P., S. Lagier, C. Baptiste, B. Marteaux, V.P. Renaud, J. Leclercq and L. Alemanno. 2008. Expression of the HEV2.1 gene promoter in transgenic *Hevea brasiliensis.* Plant Cell, Tissue and Organ Culture 94: 55-63.

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

Nakkanong, K., C. Nualsri and S. Sdoodee. 2008. Analysis of genetic diversity in early introduced clones of rubber tree (*Hevea brasiliensis*) using RAPD and microsatellite markers. Songklanakarin Journal of Science and Technology 30: 553-560.

Nayanakantha, N.M.C. and P. Seneviratne. 2007. Tissue culture of rubber: past, present and future prospects. Journal of Science (Biological Science) 36 (2): 116-125.

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

Paranjothy, K. 1974. Induced root and embryoid differentiation in *Hevea brasiliensis* in culture. 3rd International Congress of Plant Tissue and Cell Culture, University, Leicester, Abstract 67.

Paranjothy, K. and H. Gandimathi. 1976. Tissue and Organ Culture of *Hevea*. Proceedings of International Rubber Conference 59-84.

Pipatpanukul, T., S. Bunnag, P. Theerakulpisut and M. Kosittrakul. 2004. Transformation of indica rice (*Oryza sativa* L.) cv. RD6 mediated by *Agrobacterium tumefaciens*. Songklanakarin Journal Science and Technology 26: 1-13.

Priya, P., P. Venkatachalam and A. Thulaseedharan. 2006. Molecular cloning and characterization of the rubber elongation factor gene and its promoter sequence from rubber tree (*Hevea brasiliensis*): A gene involved in rubber biosynthesis. Plant Science 171: 470-480.

Rodpeawpan Kusuma. 2011. Transformation of glyphosate resistance gene into sugarcane (*Saccharum officinarum* L.). Master of Science, Kasetsart University.

Rubber Statistical Bulletin. 2007. International Rubber Study Group. UK: Wembley.

Saengruksawong, C., K. Soontorn, A. Niwat and P. Jitti. 2012. Growths and carbon stocks of para rubber plantations on Phonpisai soil series in northeastern Thailand. Rubber Thai Journal 1:1-8.

Satchuthananthavale, R. and Z.E. Irugalbandara. 1972. Propagation of callus from *Hevea* anthers. Quarterly Journal of Rubber Research Institute of Ceylon 49: 65-68.

Seneviratne, P. 1991. Micropropagation of juvenile and mature *Hevea brasiliensis*. Ph. D Thesis University of Bath, UK.

Sookmark, U., V. Pujade-Renaud, H. Chrestin, R. Lacotem, C. Naiyanetr and M. Seguin. 2002. Characterization of polypeptides accumulated in the latex cytosol of rubber trees affected by the tapping panel dryness syndrome. Plant and Cell Physiology 43: 1323-1333.

Sushamakumari, S., M.P. Asokan, P. Anthony, K.C. Lowe, J.B. Power and M.R. Davey. 2000. Plant regeneration from embryogenic cell suspension-derived protoplasts of rubber. Plant Cell, Tissue and Organ Culture 61: 81-85.

Te-chato, S. and A. Muangkaewngam. 1992. Tissue culture of rubber *in vitro* micropropagation of rubber. Songklanakarin Journal of Science and Technology 14: 123-132.

Te-chato, S. and M. Chartikul. 1993. Tissue culture of rubber: certain factors affecting callus formation from integument seed. Songklanakarin Journal of Science and Technology 15: 227-233

Te-chato, S., P. Suranilpong and S. Chuenjit. 1995. Screening of rubber callus resistant to phytophthora leaf fall agent. Journal of Science and Technology 17: 7-16.

Thu, T.T., T.T.X. Mai, E. Deade, S. Farsi, Y. Tadesse, G. Angenum and M. Jacobs. 2003. *In vitro* regeneration and transformation of pigeonpea (*Cajanus cajan* L. Mills P). Molecular Breeding 11: 159-168.

Venkatachalam, P, A. Thulaseedharan and K. Raghothama. 2007. Identification of expression profiles of tapping panel dryness (TPD) associated genes from the latex of rubber tree (*Heavea brasiliensis* Muell. Arg.). Planta 266: 499-515.

Vinod K,S. and M.V. Rajam. 2004. Polyamines enhance *Agrobacterium tumefaciens* *vir* gene induction and T-DNA transfer. Plant Science 168: 475-480.

Wang, Z., H. Wu, X. Zeng, C. Chen and Q. Li. 1984. Embryogeny and origin of anther plantlet of *Hevea brasiliensis.* Chinese Journal of Tropical Crops 5: 9-13.

Wilson, H.M. and H.E. Street. 1975. The growth, anatomy and morphogenetic potential of callus and cell suspension cultures of *Hevea brasiliensis*. Annals of Botany 39: 671-682.

Yang, L., F.L. Fu and W.C. Li. 2011. T-DNA integration patterns in transgenic plants mediated by *Agrobacterium tumefaciens*. Hereditas 33: 1327-1334.

Yang, Y., M. Bao and G. Liu. 2010. Factors affecting *Agrobacterium*-mediated genetic transformation of embryogenic callus of *Parthenocissus tricuspidata* Planch. Plant Cell, Tissue and Organ Culture 102: 373-380.

Zhang, H., Q.M. Huang and J. SU. 2010. Development of alfalfa (*Medicago sativa* L.) regeneration system and *Agrobacterium*-mediated genetic transformation. Agricultural Sciences 9: 170-178.

Zheng, S.J., L. Khrustaleva, B. Henken, E. Jacobsen, C. Kik and F.A Kren. 2001. *Agrobacterium tumefaciens*-mediated transformation of *Allium cepa* L.: the production of transgenic onions and shallots. Molecular Breeding 7: 101-115.

Zhao, Z.Y., T. Cai, L. Tagliani, M. Miller, N. Wang, H. Pang, M. Rudert, S. Schroeder, D. Hondred, J. Seltzer and D. Pierce. 2000. *Agrobacterium* mediated sorghum transformation. Plant Molecular Biology 44: 789-798.

Zhao, F.X., S.W.Chen, A. Perl, R. Dai, H.Y. Xu and H.Q. Ma. 2011. The establishment of an *Agrobacterium*-mediated transformation platform for the non-embryogenic calli of *Vitis vinifera* L. Agricultural Sciences 10: 686-694.

Zhou, Q.N., Z.H. Jiang. T.D. Huang, W.G. Li, A.H. Sun, X.M. Dai and Z. Li. 2010. Plant regeneration via somatic embryogenesis from root explants of *Hevea brasiliensis*. African Journal of Biotechnology 9: 8168-8173.

APPENDICES

Appendix A

Table 1 Composition of nutrition of Murashige and Skoog (MS)

Components	Volume (mg/l)
Macro elements	
NH_4NO_3	1,650.000
KNO_3	1,900.000
KH_2PO_4	170.000
$CaCl_2.2H_2O$	440.000
$MgSO_4.7H_2O$	370.000
Micro elements	
KI	0.830
H_3BO_3	6.200
$MnSO_4.H_2O$	16.900
$ZnSO_4.7H_2O$	10.600
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.250
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	27.800
Na_2EDTA	37.300
Organic compounds	
Myo-inositol	100.000
Nicotinic acid	0.500
Pyridoxine HCl	0.500
Thaiamine HCl	0.100
Glycine	2.000
Sucrose	30,000.00
Agar	7,500.00
pH = 5.7	

Appendix B

Table 1 Composition of nutrition of Lysogeny Broth medium (LB)

Components	Volume (g/l)
Bacto tryptone	10
Bacto yeast extract	5
NaCl	5
Bacto agar	15
pH = 7.0	

Appendix C

Preparation of solution buffers and reagents for molecular markers

1. DNA isolation by the modified CTAB method

1.1 CTAB (Hexadecyl trimethyl-ammonium bromide) buffer, 100 ml

10 ml of 1 M Tris HCl pH 8.0

8.12 g of $NaCl_2$

4 ml of 0.5 M Na_2EDTA (pH 8.0)

1 g of PVP-40

20 g of CTAB (cetyltrimethyl ammonium bromide)

Bring total volume to 100 ml with ddH_2O. Add 2 g of CTAB and put in the water bath at 60°C for 15-20 minutes to dissolve the CTAB (Don't shake the solution – the detergent will bubble up too much). Sterilize using an autoclave. Prior to starting extraction, add 2% β-mercaptoethanol in the buffer. Once these have been added the shelf life of the buffer is only 2-3 days.

1.2 1.0 M Tris-HCl (pH 8.0)

121.1 g of Tris-HCl

Dissolve in about 700 ml of H2O. Bring pH down to 8.0 by adding concentrated HCl.

1.3 TE buffer

500 µl of 1.0 M Tris-HCl (pH 7.5)

200 µl of 0.25M Na_2EDTA (pH 7.0)

Adjust the volume to 500 ml with distilled water and sterilize using an autoclave.

1.4 5X TAE buffer

121.1g of Tris Base

28.5 ml of Acetic acid

50.0 ml of 0.5M Na2EDTA (pH 8.0)

Adjust the volume to 500 ml with distilled water and sterilize using an autoclave. To make 1X TAE buffer before using.

1.5 5X TBE buffer

216 g of Tris Base

110 g of Boric acid

80 ml of 0.5M Na2EDTA (pH 8.0)

Adjust the volume to 4 L with distilled water and sterilize using an autoclave. To make 0.5X TBE buffer before using.

2. Histochemical detection of *gus* gene

X-Gluc solution ：

0.2 M NaHPO4， pH 7.0	100 ml
0.2 M $Na_2HPO4 \cdot 7H_2O$	62 ml (53.614 g)
0.2 M $NaH_2PO4 \cdot H_2O$	38 ml (27.598 g)
DI H_2O	94 ml
0.1 M $K_3[Fe(CN)_6] \cdot 3H_2O$	1 ml (6.586 g)
0.1 M $K_4[Fe(CN)_6] \cdot 3H_2O$	1 ml (8.448 g)
0.5 M Na_2EDTA	4 ml (93.06 g)
200 mg X-Gluc	
sodium phosphate buffer	50 ml
Triton-x	20 ul

Kept in refrigerator at 4 ^{o}C

3. Dot blot hybridization and southern blot PCR hybridization

3.1 Hybridization solution (10 ml)

5X SSC (stock 20X SSC)	10 ml
0.1 % N-lauroylsarcosine (stock 5 % N-lauroylsarcosine)	0.2 ml
0.02 % SDS (stock 20% SDS)	0.01 ml
1X blocking solution (stock 10x blocking solution)	0.1 ml

3.2 Low stringency buffer (200 ml)

2X SSC (stock 20X SSC)	20 ml
0.1% SDS (stock 20% SDS)	1 ml

3.3 High stringency buffer (200 ml)

0.1X SSC (stock 20X SSC)	5 ml
0.1% SDS (stock 20% SDS)	1 ml

3.4 Washing buffer (500 ml)

1X maleic acid buffer	498.5 ml
tween 20	1.5 ml

3.5 Blocking solution (10 ml)

10X blocking solution	1 ml
1X maleic acid buffer	9 ml

3.6 Detection buffer (2000 ml)

0.1 M Tris-HCl	350.64 g
0.1 M NaCl	242.2 g

Appendix D

PCR reaction

Table 1 Preparation of solution of PCR reaction (1X/20 µl)

Components	Concentration	Volume	Final concentration
PCR buffer	10X	2 µl	1X
$MgCl_2$	50 mM	0.8 µl	2 mM
dNTP mix	1 mM	4.0 µl	200 µM
F -Primer	50 µM	0.1 µl	0.25 µM
R- Primer	50 µM	0.1 µl	0.25 µM
Taq polymerase	2 U/µl	0.5 µl	1 U
DNA template		1 µl	
dH_2O		11.5 µl	
Total		20	

Table 2 PCR reaction profile

	Temperature	Time		
	94°C	2	min	30 cycles
Denaturing	94°C	40	sec	
Annealing	60°C	30	sec	↓
Extension	72°C	1	min	
	72°C	5	min	

Table 3 DNA sequences of primers

	Primer	Sequences
gus	Forward	5'-CTGCGACGCTCACACCGATAC-3'
	Reward	5'-TCACCGAAGTTCATGCCAGTCCAG-3'
EPSPs	Forward	5'-CCATTCCGCTCGAGATGGCA CAAATTAACAACATGGC-3'
	Reward	5'ATCCACCGCTCGAGCGGTCATCAGGCAGCCTTC GTAT-3'

,

PAPER 1

Improvement Tissue Culture Technique of Para Rubber for Gene-Transformation

PAPER 2

The Establishment of *Agrobacterium*-Mediated Gene Transformation in Rubber Tree through Organized Explants

PAPER 3

The Effect of *Agrobacterium* Densities and Inoculation Times on Gene Transformation Efficiency in Rubber Tree

Vitae

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List of Publications and Proceedings

1. Publications

Kalawong, S. and Te-chato, S. 2012. Factors affecting gene transformation in embryogenic callus of oil palm by bombardment technique. Journal of Agricultural Technology 8: 2373-2384.

Kalawong, S. and Te-chato, S. 2014. *In vitro* shoot induction of rubber tree using microcutting technique. Songklanakarin Journal of Plant Science 3: 13-19.

Kalawong, S., Srichuay W., Sirisom Y. and Te-chato S. 2014. The Establishment of *Agrobacterium*-Mediated Gene Transformation in Rubber Tree through Organized Explants. Journal of Agricultural Technology 10: 493-503.

Kalawong, S., Srichuay W. and Te-chato S. 2014. The effect of *Agrobacterium* densities and inoculation time on gene transformation efficiency in rubber tree. African Journal of Biotechnology 13: 2321-2329.

Kalawong, S. and Te-chato, S. 2014. A study on proliferation of anther-derived cell suspension culture of rubber tree. Agricultural Science Journal. (in press)

2. Proceedings

Kalawong, S. and Te-chato, S. 2012. *Agrobacterium*-mediated Gene Transformation in Rubber Tree. Innovations in Agriculture and Natural Resource Faculty of Natural Resources, Prince of Songkla University, 13rd November 2012. (Oral presentation)

Kalawong, S. and Te-chato, S. 2013. A study on proliferation of anther-derived cell suspension culture of rubber tree. The 12th National Toward AEC Under Climate Changes. Bangkok International Trade & Exhibition Centre, Bangkok, Thailand. 9-12 May 2013. (Poster)

Kalawong, S. and Te-chato, S. 2013. *In vitro* shoot induction of rubber tree using

microcutting technique. The 1st Plant Science Congress “From Plant Science to AEC”. Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Thailand. 13-14 August 2013. (Oral presentation)

Kalawong, S. 2014. Improvement Tissue Culture Technique of Rubber Tree for Gene Transformation. 1st USM-PSU *Hevea* Tissue Culture workshop, School of Biological Sciences, USM, Malasia, 20th January 2014. (Oral presentation)

Kalawong, S. and Te-chato, S. 2014. *In vitro* shoot induction of rubber tree using microcutting technique. The 2nd Plant Science Congress “Plant science research make values to sustainable agriculture”. Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Thailand. 13-14 August 2014. (Poster)

Kalawong, S. 2014. Microcutting as a Tool for Propagation and Genetic Transformation in Rubber Tree. 2014 International Conference on Rubber, Thaksin University, Phatthalung campus, Thailand, 28-30th August 2014. (Poster)