Biolistic Transformation in Oil Palm (*Elaeis guineensis* Jacq.)

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ชื่อวิทยานิพนธ์ การถ่ายโอนยีนโดยวิธี biolistic เข้าสู่ปาล์มน้ำมัน (*Elaeis guineensis* Jacq.)

ผู้เขียน นางคันธมาทน์ กาญจนภูมิ

สาขาวิชา ชีววิทยาโมเลกุลและชีวสารสนเทศ

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บทคัดย่อ

ปาล์มน้ำมัน (*Elaeis guineensis* Jacq.) เป็นพืชที่เพาะเลี้ยงเนื้อเยื่อและถ่ายโอนยีนยากชนิดหนึ่ง เมื่อศึกษาผลของไคโตซานและ TDZ ในการชักนำให้เกิดต้นใหม่จากแคลลัสปาล์มน้ำมัน พบว่าไคโตซาน 15 มก/ล ในอาหารเพาะเลี้ยงสูตร MS (Murashige and Skoog, 1962) ทั้งที่มีและไม่มี 2, 4-D 5 มก/ล ร่วมด้วย สามารถส่งเสริมการชักนำให้เกิดต้นใหม่จากแคลลัสปาล์มน้ำมันได้แม้ว่าอัตราการชักนำให้เกิดต้นใหม่ยังค่อนข้างต่ำ ส่วนระบบถ่ายโอนยีนในปาล์มน้ำมันโดยวิธี biolistic ที่มีประสิทธิภาพทำโดยใช้ยีน *EgTCTP* (*Elaeis guineensis Translationally Controlled Tumor Protein*) และพลาสมิดเวคเตอร์ pCAMBIA 1302 ซึ่งบรรจุ green fluorescent protein (*mgfp5*) ที่ทำหน้าที่เป็นยีนรายงานผล และยีน hygromycin phosphotransferase (*hpt*) ที่ทำหน้าที่เป็นยีนคัดเลือก ภายใต้การควบคุมของโปรโมเตอร์ cauli-flower mosaic virus (CaMV) 35S ชิ้นส่วนปาล์มน้ำมันที่นำมาใช้ศึกษาการยิงยีนมี 3 ชนิดคือ คัพภะแก่จัด ต้นอ่อน และแคลลัส สภาวะที่ใช้ควบคุมการยิงยีนคือ ระยะทางจาก rupture disk ถึง macro-carrier 11 มม ระยะทางจาก macrocarrier ถึงเนื้อเยื่อเป้าหมาย 90 มม ใช้ผงทองขนาด 1 ไมครอนเป็น microcarrier ระดับแรงดันก๊าซฮีเลี่ยมที่ใช้ยิงยีนคือ 850, 1100, 1300 และ 1550 psi คัดเลือกชิ้นส่วนพืชที่ผ่านการยิงยีนในอาหารเพาะเลี้ยงที่มี hygromycin 50 มก/ล นาน

2 เดือน พบว่าแรงดันก๊าซฮีเลี่ยมที่เหมาะสมในการใช้ยิงยีนในคัพภะแก่จัด และต้นอ่อนคือ 850 และ 1550 psi ตามลำดับ ในขณะที่ประสิทธิภาพการถ่ายโอนยีนในแคลลัส100% เกิดขึ้นที่ทั้งระดับ 850 และ 1550 psi อย่างไรก็ตามที่ระดับแรงดันก๊าซ 1100 และ 1300 psi ยังคงเหมาะสมต่อการถ่ายยีนในแคลลัสเมื่อเปรียบเทียบกับคัพภะแก่จัดและต้นอ่อน อัตราการเจริญเติบโตของแคลลัสหลังจากที่ได้รับการถ่ายยีนที่ 1100 psi นาน 6 เดือนคือ 0.51 มก/วัน มีความแตกต่างอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม ขณะที่อัตราการเจริญเติบโตของต้นอ่อนปาล์มน้ำมันหลังจากได้รับการถ่ายยีนที่ 1300 psi นาน 1 ปี 6 เดือน จึงจะพบความแตกต่างอย่างมีนัยสำคัญ นอกจากนี้ไม่พบความแตกต่างอย่างมีนัยสำคัญในคัพภะแก่จัดที่ได้รับการถ่ายยีนทุกระดับแรงดันก๊าซ แคลลัสของปาล์มน้ำมันจึงเป็นเนื้อเยื่อเป้าหมายที่เหมาะสมต่อการถ่ายโอนยีนโดยวิธี biolistic ที่แรงดัน 1100 psi การวิเคราะห์ทางเนื้อเยื่อวิทยาช่วยยืนยันว่า ความหนาแน่นของเซลล์ปาล์มน้ำมันที่ได้รับยีน *EgTCTP* สูงกว่าความหนาแน่นของเซลล์ในกลุ่มควบคุมถึง 4 เท่า นอกจากนี้พบว่า ยีน *EgTCTP* ช่วยในการเพิ่มจำนวนและการเกิดเป็นต้นใหม่ใน protocorm like-bodies (PLBs) ของกล้วยไม้สกุล *Phalaenopsis* จากการตรวจสอบการเรืองแสงสีเขียวของยีน *mgfp5* ทั้งในปาล์มน้ำมันและกล้วยไม้ที่ได้รับการถ่ายยีน *EgTCTP* พบจุดเรืองแสงสีเขียวในชิ้นส่วนพืชที่ผ่านการยิงยีนทุกชนิด แต่จะไม่พบจุดเรืองแสงสีเขียวในกลุ่มควบคุมที่ปราศจากเวคเตอร์ pCAMBIA 1302 นอกจากนี้เทคนิค PCR สามารถใช้เพื่อพิสูจน์ว่า *EgTCTP* เข้าไปรวมอยู่ในจีโนมของพืชที่ได้รับการถ่ายยีนจริง จากข้อมูลทั้งหมดที่ค้นพบกล่าวได้ว่า *EgTCTP* ทำหน้าที่คล้ายคลึงกับ TCTP ที่พบในสิ่งมีชีวิตอื่นทั่วไป และนับว่าเป็นรายงานฉบับแรกที่นำยีน *EgTCTP* เข้าสู่ปาล์มน้ำมันและผลิตพืชที่ได้รับการถ่ายยีน *EgTCTP* แสดงให้เห็นว่า *EgTCTP* สามารถใช้ถ่ายโอนยีนโดยวิธี biolistic ในปาล์มน้ำมันเพื่อประโยชน์ในแง่ของการเจริญเติบโตและการเกิดเป็นพืชต้นใหม่

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ABSTRACT

Oil palm (*Elaeis guineensis* Jacq.) is known as one of the most recalcitrant species for tissue culture and genetic transformation. The effects of chitosan and TDZ to improve plant regeneration of oil palm callus were studied. It was found that MS medium supplemented with 15 mg/l chitosan either with or without 5 mg/l 2, 4-D could induce shoot regeneration in oil palm callus even though the regeneration rate was quite low. An efficient genetic transformation system via biolistic method in oil palm was established using the *EgTCTP* (*Elaeis guineensis translationally controlled tumor protein*), the pCAMBIA 1302 plasmid vector containing the green fluorescent protein (*mgfp5*) as reporter gene, and the hygromycin phosphotransferase (*hpt*) gene as selectable marker under the control of cauliflower mosaic virus (CaMV) 35S promoter. Three types of oil palm explants namely mature embryos, young seedlings and embryogenic callus were bombarded under the same conditions as follows: rupture disk to macrocarrier distance, 11 mm; macrocarrier to target tissue, 90 mm and using 1 µm gold particles as microcarrier. Four helium pressure levels 850, 1100, 1300, and 1550 psi were tested. Then, the bombarded explants were selected on MS medium containing 50 mg/l hygromycin cultured for 2 months. Results revealed that the optimum helium pressure for DNA delivery into mature embryos and seedlings was 850 and 1550 psi, respectively. While the pressure for callus was achieved at both 850 and 1550 psi levels with 100% transformation efficiency. However, the pressure levels at 1100 and 1300 were also efficient for callus bombardment compared to mature embryos and seedlings. Growth of callus after bombardment at 1100 psi for 6 months was significantly different from the control while growth of seedlings after bombardment at 1300 psi was found significantly different after 18 months. Furthermore no significant differences were found in

mature embryos at all levels of helium pressure. Therefore it can be concluded that the suitable helium pressure for oil palm callus bombardment was 1100 psi and embryogeneic callus was a suitable target tissue for the study of the role of *EgTCTP* when the transformation was carried out via Biolistic method. In addition, histological analysis was also confirmed that the cell number of transformants was 4-fold higher in cell density than the control groups. In the parallel study with *Phalaenopsis*, a monopodial orchid, the proliferation and regeneration capacity of *EgTCTP* transformed protocorm like bodies also increased suggesting the role of *EgTCTP* gene in this monocot. To monitor the expression, presence and integration of the transgenes in the putative transformants of both oil palm and orchid explants, GFP green fluorescence spots of *mgfp5* gene were detected by confocal laser scanning microscope in all explants but none in the controls without pCAMBIA 1302 vector. For stability assay, the presence of transgenes in the hygromycin resistant explants to indicate that the transgene was integrated into the genome of transformants was confirmed by PCR. Taking all these findings into account, *EgTCTP* showed the most likely functions in a manner similar to other known TCTP. This is the first report to introduce *EgTCTP* gene into oil palm and produce TCTP transformants. It seems that *EgTCTP* gene is a good candidate to assist the biolistic transformation of oil palm and provides benefits for growth and regeneration.

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Kantamaht Kanchanapoom

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LIST OF ABBREVIATIONS AND SYMBOLS

2, 4-D	=	2, 4-dichloro-phenoxyacetic acid
AC	=	activated charcoal
AIL	=	AINTEGUMENTA-like
bp	=	base pair (s)
Bt	=	*Bacillus thuringiensis*
°C	=	degree celcius
$CaCl_2$	=	calcium chloride
CaMV	=	cauliflower mosaic virus
CLSM	=	confocal laser scanning microscope
CpTI	=	*cowpea trypsin inhibitor*
CW	=	coconut water
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
EgTCTP	=	*Elaeis guineensis Translationally Controlled Tumor Protein*
ESTs	=	expressed sequence tags
et al.	=	and others
FAA	=	formaldehyde–acetic acid–alcohol
FAO	=	Food and Agricultural Organization
g	=	gram
gfp	=	green fluorescent protein
gus	=	β-glucuronidase
h	=	hour (s)
HCl	=	hydrochloric acid
Hg	=	mercury
hpt	=	hygromycin phosphotransferase

Abbreviations and Symbols (Continued)

HRF	=	histamine releasing factor
HYG	=	hygromycin
i.e.	=	id. est, for example
kb	=	kilobase (s)
l	=	liter
M	=	molarity
mM	=	millimolar
mg	=	milligram
min	=	minute (s)
ml	=	milliliter
mm	=	millimeter
MS	=	Murashige and Skoog
*npt*II	=	neomycin phosphotransferase II
μg	=	microgram
μl	=	microliter
μM	=	micromolar
NaOH	=	sodium hydroxide
ng	=	nanogram
nm	=	nanometer
ORF	=	open reading frame
PCR	=	polymerase chain reaction
ppt	=	part per thousand
PLBs	=	protocorm like-bodies
RIP	=	*ribosome-inactivating protein*
RNA	=	ribonucleic acid
RNase	=	ribonuclease

Abbreviations and Symbols (Continued)

rpm	=	round per minute
sec	=	second
TAE	=	Tris-acetate-EDTA
TCTP	=	Translationally controlled tumor protein
TDZ	=	phenyl-N^{1}-1, 2, 3,-thiadizol-5-ylurea
Ti	=	tumor-inducing
U	=	unit (s)
UV	=	ultraviolet
v/v	=	volume per volume
w/v	=	weight per volume

CHAPTER 1
INTRODUCTION

1.1 Background and Rationale

During the past decade transgenic technology is currently standard practice in many crops. In 2008, the acreage and the number of farmers using biotech crops is rising with 309 million acres planted in 2008 compared with 282 million in 2007. Also this year, the United States planted nearly half of those acres with 154 million acres and several African countries began planting biotech crops. According to the record, a 13.3 million farmers in 25 countries, of which 90% are resource-poor farmers in 15 developing countries are using agricultural biotechnology (James, 2008). This progress can be attributed to developments in molecular genetics, plant transformation and regeneration techniques. Moreover, the doubt processes involved in DNA recombination should be discover from the update research knowledge.

Currently, there is very strong growth in the demand for vegetable oils. A new demand for using as bio-fuel also needs to be taken into account. Palm oil is the second largest source of edible oil in the world but it is a perennial monocot tree with a long generation period. Thus, the choice of target tissue type in oil palm becomes critical to shorten the time taken to produce transgenic oil palm. In addition, the translationally controlled tumor protein (TCTP) has the potential to be used as a transgene for oil palm to promote plant growth due to the function of *TCTP*s in animals related to proliferation and differentiation (Hinojosa-Moya *et al*., 2008).

In general, almost significant crop species such as soybean, cotton, maize, rice, wheat has been successfully transformed but in many species, especially oil palm, the development of highly efficient and routine transformation systems is still in progress. To date, several useful genes (*Bt*, *CpTI*, *RIP*, etc.) that mentioned in the transgenic oil palm part have been successfully engineered into oil palm with

transgenic plants produced in Malaysia. However, transformation frequencies in oil palm are still relatively low.

Unlike other crops, oil palm tissue culture is a very slow process. On average at least 18 months are required to produce complete plants from callus. Therefore, the ultimate goal of this research is to improve the transformation efficiency via biolistic methods in an appropriated oil palm explants for achieving stable gene transformation and rapid plant regeneration system. Moreover, application of the *TCTP* transgene would be done in other monocot which has a shorter life cycle such as orchids to confirm the *TCTP* role in monocotyledon plant.

1.2 Review of Literature

The taxonomic hierarchy of *Elaeis guineensis* Jacq.

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Liliopsida* – Monocotyledons
Subclass	*Arecidae*
Order	*Arecales*
Family	*Arecaceae* – Palm family
Genus	*Elaeis* Jacq. – oil palm
Species	*Elaeis guineensis* Jacq. – African oil palm

The African oil palm (*Elaeis guineensis* Jacq.), belongs to the Arecaceae, is a tropical perennial species with a long generation period. The oil palm is native to west Africa, occurring between Angola and Gambia, while the American Oil Palm, *Elaeis oleifera*, is native to tropical Central America and South America. From Wikipedia, the generic name is derived from the Greek for oil, *elaion*, while the species name refers to its country of origin. Initially, it was

first illustrated by Nicholaas Jacquin in 1763.

The arborescent monocotyledon is the typical morphology of palm. At the adult stage, palm crown consists of 40-50 open palmate leaves which produce by a single terminal meristem on average two leaves per month. The height of palm tree can reach 30 m with a 10-16 m in diameter leaf crown (Figure 1). The root system is made of a large number of fasciculate adventitious roots. Oil palm is grown for 30 months before the first yield can be harvested and it then maintains the yield continually for the next 20–30 years. The oil palm is a temporal dioecious species which displays alternate male and female flowering cycles throughout its life. The flowers are produced in dense clusters. Each of individual flowers is small with three sepals and three petals. Unlike other relatives, the oil palm does not produce offshoots. The oil palm normally propagated by sowing the seeds. The fruit maturation takes five to six months from pollination to maturity. The oil palm fruit is the source of both crude palm oil which extracted from the mesocarp and crude palm kernel oil which extracted from the kernel.

Figure 1 Botanical description of *Elaeis guineensis*

Source: caliban.mpiz-koeln.mpg.de/~stueber/koehler/

Palm oil has multiple uses, as in the production of cooking oils, margarines, soaps, detergents and as a fuel. After harvesting, the fresh fruit bunches must be delivered to the palm oil mill within 24 hours because the free fatty acid content which affects the quality of the palm oil increases significantly after the fruits are picked. From this reason, good quality palm oil should contain less than 5% of the free fatty acid content. A bunch of fruits can weigh between 10 to 40 kilograms. Typically, crude palm oil is used for biodiesel production as it can be obtained in a larger quantity than crude palm kernel oil. For every 100 kilograms of fruit bunches, typically 22 kilograms of palm oil for an edible oil and 1.6 kilograms of palm kernel oil can be extracted, used mainly for soap manufacture.

Oil palm is grown in tropical climates, particularly in Southeast Asia (Malaysia, Indonesia, and Thailand) which has suitable climatic and soil conditions. Based on the Food and Agricultural Organization (FAO) data, of the total area in the world under oil palm, 63% is in Southeast Asia, and 80% of the world's palm oil is produced in this region. The high productivity of the palm oil as high as 7,250 liters per hectare per year has made it the prime source of vegetable oil for many tropical countries. In addition, oil palm has great potentials for using as a renewable energy source to replace diesel oil. Accordingly, bioenergy has attracted major attention from policy makers, energy planners, the private sector, and the general public in recent years.

The palm oil industry will continue to grow because of many advantages from palm oils**.** The world's largest producer and exporter of palm oil today is Malaysia, producing about 47% of the world's supply of palm oil. In Thailand, oil palm is one of the most economic crop. In order to satisfy growing demand in India and China, Thailand government has recently set up a very ambitious development plan for oil palm which commonly planted in the southern region of Thailand. There are 44 companies of crude palm oil extract refineries in 9 provinces of the southern region of Thailand, with the production capacity of 841,657 tons per year. There are also 11 companies of pure palm oil refineries with a production capacity of 56,447 tons per month.

Estimated by flow cytometric analysis, the genome of oil palm is $3.4x10^9$ base pairs with a chromosome number of 2n=2x=32 (Rival *et al*., 1997). Much of the oil palm genome consists of repetitive DNA sequences (Castilho *et al.*, 2000). However, little is known about the molecular changes associated with callogenesis and embryogenesis in oil palm. In order to understand the genes expressed during the oil palm tissue culture, a large set of oil palm expressed sequence tags (ESTs) was generated in the development of oil palm tissue culture. This technique has proven to be a rapid and efficient way of obtaining information on gene diversity and mRNA expression patterns from a wide variety of tissues, cell types or developmental stages (Low *et al*., 2008).

Biotechnology was first introduced to the oil palm industry almost 30 years ago. Oil palm is generally propagated by seeds therefore a great variation in the plantation is expected due to the heterozygosity of the seedlings. In addition, seed germination of some cultivars such as *Psifera* (Shell-less; embryo rarely form) is very poor. Conventional vegetative propagation in this woody allogamous species is not possible and tissue culture has been used as a tool to overcome this impediment. Initially, tissue culture techniques were used to propagate elite oil palm clones on a large scale in the shortest possible time. To obtain this aim, the abnormal inflorescences of clonal palms were produced due to the excessive use of plant growth hormones particularly cytokinins (Jones, 1998). Following this problem, oil palm tissue culture techniques have undergone continuous improvement until now (Zamzuri *et al*., 2007). The production of clonal palms is currently more reliable with minimal abnormality feature. Therefore, it is envisaged that clonal palms would eventually replace seed-derived planting materials on a commercial scale.

Plant regeneration via *in vitro* culture has been initiated from various sources of explants (Teixeira *et al.*, 1995; Patcharapisutsin and Kanchanapoom, 1996; Sambanthamurthi *et al*., 1996; Aberlenc-Bertossi *et al*., 1999). Embryo culture has proved useful to increase the number of seedlings and it is interesting to use as a convenient source of explant for callus induction. Furthermore, embryo culture is an important prerequisite for the successful application of several *in vitro* techniques. Zygotic embryos of palms have been mainly used as explants for

induction of callus with generative capacity as in coconut palm (Fisher and Tsai, 1978; Gupta *et al*., 1984), date palm (Gabr and Tisserat, 1985; Fki *et al*., 1984), Christmas palm (Srinivasan *et al.*, 1985), sago palm (Alang and Krishnapillary, 1986), *Hyophorbe amaricaulis* palm (Douglas, 1987), *Washingtonia filifera* palm (DeMason, 1988), Canary Island date palm (Le Thi *et al*., 1999) and macaw palm (Moura *et al*., 2008). A callus-mediated plant regeneration protocol is a prerequisite and fundamental requirement for the exploitation and improvement of this plant through genetic transformation. Though most of plant regeneration in oil palm was produced through callus and cell suspension cultures but the growth of either calli or cells in cultures is very slow. Therefore most culture media were modified by the addition of auxins or cytokinins to accelerate efficient regeneration. Information concerning details of media and growth regulator amendments is still a fundamental requirement of the intense commercial production.

In the past, Touchet *et al*. (1991) reported the first establishment and maintenance of regenerable embryogenic suspension cultures from oil palm leaf derived calli. Recently, a mathematical model for the growth and conversion of somatic embryos was developed with the aim of monitoring the large scale production of oil palm microplants (Konan *et al*., 2006). As in the case for most monocots, the frequencies for complete plant regeneration from somatic embryogenesis are still inefficient (Abdullah, 2005). Therefore, in recent years, chitosan has attracted notable interest due to its biological activities such as antimicrobial, antitumor and stimulation of plant growth.

Chitosan is a natural carbohydrate polymer derived by deacetylation of chitin which consists of N-acetyl-D-glucosamine and D-glucosamine residues linked by β-1, 4 glycosidic bonds (Chibu and Shibayama, 2001). Chitosan is the main structural component of squid pens, cell walls of some fungi and shrimp or crab shells which easily degradable. In addition, chitosan has been shown to affect on the improvement of secondary metabolite production in plant cell cultures which influence the production of substances related to stress response, such as phytoalexins (Walker-Simmons *et al.,* 1983) and chitinases (Dornenburg and Knoor, 1994, O'Herlihy *et al.*, 2003) Moreover, chitosan is an exogenous elicitor which plays a role in plant resistance to pathogens, defense mechanism and

promoting plant growth, resulting in improved yields and plant health (Uthairatanakij *et al.*, 2007). Many research data in agriculture and horticulture have been collected for the effect of chitosan on promote growth of various crops such as rice (Boonlertnirun *et al.*, 2005), soybean sprouts (Lee *et al.*, 2005), vegetables (Kim, 2005) and especially on orchid cultivation which a positive effect of chitosan was observed on the growth and development of *Dendrobium phalaenopsis* orchid both in liquid and on solid medium (Nge *et al.*, 2006). However, chitosan has inconsistent effects on growth and development of mature orchid plants. Nevertheless, the mechanism of action of chitosan on plant growth is still unclear. Possibly, chitosan may induce a signal to synthesize plant hormones such as gibberellins and may enhance growth and development by some signalling pathway related to auxin biosynthesis via a tryptophan-independent pathway (Uthairatanakij *et al.*, 2007). Moreover, Bolto *et al.* (2004) found that chitosan can increase the microbial population and transforms organic nutrient into inorganic nutrient, which easily absorbed by the plant roots. It is indicated that chitosan which put in the soil acting as the carbon source for microbes to accelerate the transformation process of organic matter into inorganic matter and assist the absorption of plant root system. Hence it was possible to study on the effect of chitosan with the objective of developing a protocol for the regeneration of oil palm from callus.

To improve crop productivity and added the economic value of oil palm products, genetic engineering which has made possible free movement of genetic materials from one organism to another one could be done for better oil palm explants. Currently, there are many techniques for plant transformation such as electroporation, micro-injection, *Agrobacterium*-mediated transformation and biolistic transformation. The commonly used methods for obtaining transgenic oil palm are *Agrobacterium*-mediated transformation and biolistic transformation.

Agrobacterium-mediated transformation

For the first method, *Agrobacterium tumefaciens* is a soil bourne gram negative bacterium which belongs to the family Rhizobiaceae, which has a unique

ability to introduce part of its DNA (Ti-plasmid; tumor-inducing plasmid) into the nucleus of plant cells. The specific segment of the Ti-plasmid, T-DNA, is integrated into the plant genome and leads to the formation of a so-called crown gall (Figure 2). T-DNA might be engineered by initial disarming (removal of the bacterial tumorigenic genes contained in the T-DNA) and insert a selectable marker and genes of interest. Many plant species have been transformed using T-DNA-derived vectors. In the laboratory, genetically engineered *Agrobacteria* are co-cultured (inoculated) with plant tissue, and then transfer part of the transgenes into plant cells. *Agrobacterium*-mediated transformation method often results in a single-copy or low-copy integration of full length T-strands carrying intact copies of the transgenes.

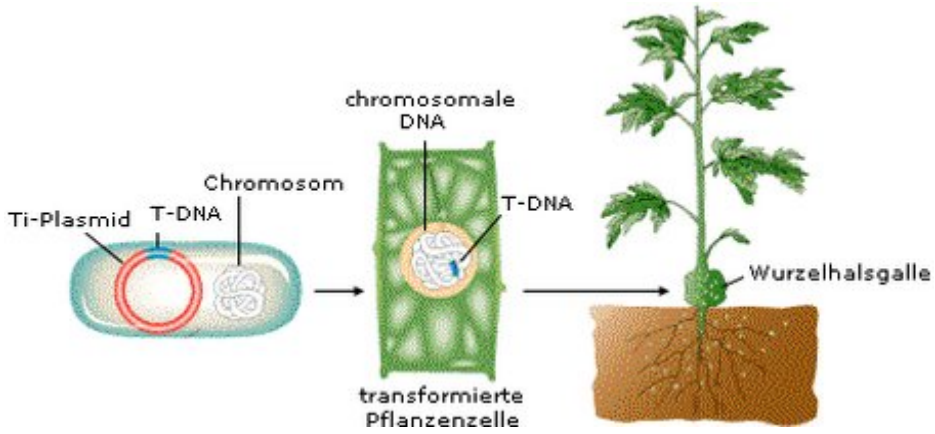


Figure 2 *Agrobacterium* inserts a T- DNA into the nucleus of a plant cell.

Source : http://www.gmo-safety.eu/en/maize

This method is advantageous in term of the minimal equipment cost as well as regarding its high efficiency, and have been well established for most dicotyledonous (Yu *et al*., 2007; Rothrock *et al*., 2007; Bhattacharjee *et al*., 2008; Xu *et al*., 2009), being natural hosts of *A. tumefaciens*, and some monocotyledonous species (Samiphak and Siwarungson, 2006; Nguyen *et al*., 2007; Krishnan, *et al*., 2009).

Although the oil palm was perceived to be recalcitrant to *Agrobacterium* in the pioneer period, the susceptibility of oil palm tissues to *Agrobacterium* infection was obtained with a transformation frequency of 11.1 to 64%. However, in more recent study, the transformation frequency was higher when using a superbinary vector instead of the normally used binary vector (Abdullah, 2005). From this study, it was shown that pre-culture of target tissues is the key to successful *Agrobacterium*-mediated gene transformation in oil palm. Nowadays, many researcheres have further improved the *Agrobacterium*-mediated gene transfer system in oil palm.

Biolistic transformation

Another method, the biolistic process, first reported by Sanford *et al*. (1987), is a physical method for DNA introduction and the biological incompatibilities associated with *Agrobacterium* are avoided. The other names of biolistic transformation are particle bombardment, microprojectile bombardment, particle acceleration, particle gun or gene gun. The basic principle of the biolistic method is to use high velocity microprojectiles to penetrate the outer tissue layers in order to introduce genetic materials into living cells, which then survive to express the introduced gene. By this way, the DNA-coated particles can end up either near or in the nucleus, where the DNA comes off the particles and might be integrated into plant chromosomal DNA.

Different commercially available devices, Helios Gene Gun System or Biolistic PDS-1000/He System–BIO-RAD (Figure 3) provide the motive force (helium pulse) for launching and delivering of DNA-coated particles into virtually any target such as organ, tissue or single cell. Fine tuning of motive force by changing the helium pressure range results in changed velocity and final distribution of microparticles in target affect the transformation success. Also, varying the particle density and size can affect bombardment efficiency.

Nowadays, the biolistic technique has been used in many diverse plant species in order to obtain the desirable transgenic plants eg. marigold (Vanegas *et al.,* 2006), rice (Riaz *et al*., 2006), barley (Tobias *et al*., 2007), grapevine (Amar *et al*., 2007), white spruce (Lachance *et al*., 2007), orchids (Suwanaketchanatit *et al*.,

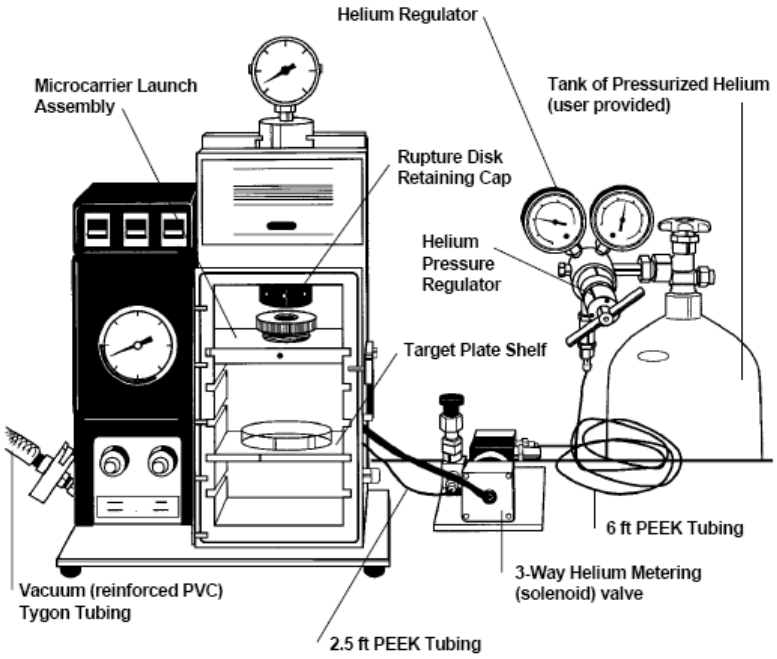


Figure 3 Unit component of the Biolistic PDS-1000/He Particle delivery system.

Source: Biolistic® PDS-1000/He Particle Delivery System Catalog

2007; Yee *et al*., 2008), cowpea (Ivo *et al*., 2008), hop (Batista *et al*., 2008). For the advantages of the biolistic transformation, it has a wide range of uses today and can be used on many organisms such as bacteria, yeasts, and mammalian cell lines, particularly those which have previously been difficult or impossible to transfect such as non-dividing cells or primary cells. Interestingly, the transformation does not apply only to unicellular organisms but also whole objects such as leaves or entire animals. Moreover, this method has been particularly useful for chloroplasts as well because no bacteria or viruses were known to infect chloroplasts (Carsono and Yoshida, 2008). To the contrary, there are some limitations in this direct gene transfer method; the transformation efficiency is low, high number of the transgene copies integrated into the plant genome, low survival of bombarded cells caused by mechanical damages, intracellular target (cytoplasm, nucleus, vacuole, plastids, etc.)

is random and high costs of the equipment and single parts especially gold microcarriers.

The biolistic method was initially chosen as a method for oil palm transformation as it has been the most successful method for monocotyledons (Parveez *et al.*, 2000; Lee *et al.*, 2006; Majid and Parveez, 2007; Omidvar *et al.*, 2008). However the transformation efficiency was still rather low. There are several variables that must be controlled in order to attain maximal transformation efficiency. Since the transformation efficiency has been proportional to the efficiency of the tissue culture and gene transfer systems (Birch, 1997), the parameter optimization involved in transformation often requires to achieve high rates of DNA expression. Studies on the physical and biological parameter optimization (Parveez *et al*., 1997; 1998), efficient target tissue (Abdullah *et al.,* 2005; Kanchanapoom *et al*., 2008) and promoters influence (Chowdhury *et al.,* 1997; Ramli and Abdullah, 2003; Masani and Parveez, 2008; Omidvar *et al.*, 2008) in assaying genetic transformation events have been reported for oil palm.

Transgenic oil palm

In 2000, the Palm Oil Research Institute of Malaysia (PORIM) which merged with the Palm Oil Licensing Authority (PORLA) to form the Malaysian Palm Oil Board (MPOB) deals with all aspects of oil palm and palm oil development in Malaysia. To date, there are many intensive researches on the transgenic oil palm plant as to increase the degree of unsaturation in palm oil (Masani and Parveez, 2008), oil quality (Wahid *et al.*, 2005), insect resistance (Lee *et al.*, 2006), fungal resistance (Yeun, 2000) and the synthesis of thermoplastics (Omidvar *et al.*, 2008). Consequently, several useful genes such as *Bacillus thuringiensis* (*Bt*) toxin, *cowpea trypsin inhibitor* (*CpTI*), *chitinase, ribosome-inactivating protein* (*RIP*) have been successfully engineered into oil palm by transformation (Lee *et al*., 2006).

New approaches are being tested to shorten the production time of transgenic oil palms. For the gene of interest, the TCTP is a highly conserved protein in all species studied and one of the identified growth-related proteins in plants. The *TCTP* gene was found about 20 years ago by three groups interested in

translationally regulated genes. They named this protein P21, Q23 and P23, respectively (reviewed in Gachet *et al.*, 1999). At this time the name 'translationally controlled tumour protein' was coined (Gross *et al*., 1989), based on the fact that the cDNA was cloned from a human tumor and on the observation that TCTP is regulated at the translational level. Moreover, TCTP was found to be expressed in healthy animal tissues at both the transcriptional and post-transcriptional levels (Sanchez *et al*., 1997; Xu *et al*., 1999). TCTP nowadays has attracted the attention of an increasing number of researchers interested in various biologically and medically relevant processes. This is largely due to the fact that TCTP levels are highly regulated in response to a wide range of extracellular stimuli, and therefore, the *TCTP* gene might be important for cell cycle progression and malignant transformation. In addition, TCTP was shown to display an extracellular function as a histamine release factor and to have anti-apoptotic activity. These findings led the authors to suggest yet other names for this protein, such as histamine releasing factor (HRF). Therefore, TCTP is a growth related protein believed to play an important role in cell growth and cell division in general (Bommer and Thiele, 2004).

Recently, a plant calcium binding *TCTP* gene has been successfully transformed into a tobacco plant. From this work, transgenic tobacco which transformed with a gene encoding TCTP grow about 30% faster than the parental plants during the juvenile growth stage (Kang *et al*., 2005). Accordingly, the ultimate goal of this research is to manipulate the faster growth of oil palm explants to reduce the long generation time of this perennial tree. For this purpose, transgenic oil palm overexpressing *TCTP* should grow faster than the non-transformed explants. In accordance with this, *TCTP* was cloned from young leaves of *E. guineensis* (*EgTCTP*). It consists of 507 base pairs and the open reading frame (ORF) codes for a protein of 168 amino acids. A calculated molecular mass is 19.2 kDa with predicted pI of 4.67. *EgTCTP* has a high homology (78%-85% identity) at the amino acid level compared to other TCTPs from *Hevea brasilliensis*, *Glycine max*, *Oryza sativa*, *Medicago sativa* and the P23 protein from *Solanum tuberosum*. (Nakkaew *et al.*, 2007)

To confirm successful transformation, a suitable selection procedure is needed. At least two essential genes, a reporter gene and a selectable marker, are used to incorporate into the plant cell. The reporter genes are visible markers, which can be used to investigate foreign gene expression in transformed cells. Currently, many reporter genes have been used for plant transformation including β-glucuronidase (*gus*) gene (Jefferson *et al.*, 1987), the firefly luciferase gene, the anthocyanin pigmentation gene and the green fluorescent protein (*gfp*) gene from the jellyfish *Aequorea victoria* (Hraška *et al.*, 2006). When compared the effectiveness of using the GFP and GUS as reporter genes for early detection of transgene expression in explants subjected to particle bombardment and *Agro-bacterium*-mediated transformation, the results indicate that *gfp* gene is superior to *gus* gene in following transgene expression (Jeoung, *et al*., 2002). GFP can be easily visualized under UV/blue light without any additional substrate. Otherwise, it has been widely used to monitor transgene expresssion and protein localization in organisms.

From the previous study, various GFP plasmids were bombarded into oil palm cultures to evaluate its feasibility in oil palm transformation system (Majid and Parveez, 2007; Kanchanapoom *et al*., 2008; Omidvar, *et al*., 2008). The results showed that GFP driven by Cauliflower Mosaic Virus (CaMV) 35S promoter produced the highest fluorescent signal in oil palm cultures. Therefore, GFP is an attractive reporter for non-destructive monitoring and used to confirm successful transformation in this study.

As a selective marker, antibiotic or herbicide resistance genes are commonly used. In addition, the most widely used marker genes include *npt*II (neomycin phosphotransferase II) gene and *hpt* (hygromycin phosphotransferase) gene which confer resistance to kanamycin and hygromycin (HYG), respectively. The oil palm target tissues were very sensitive to HYG, Basta and phosphinotrycin (Abdullah *et al.*, 2005; Parveez *et al.*, 2007). These selection agents could inhibit the growth of immature embryos at a very low concentration when compared to kanamycin, geneticin, paromomycin. Furthermore, the optimal concentration of HYG used was 50 mg/l since all immature oil palm embryos exposed to more than 50 mg/l HYG were dead within 8 weeks (Abdullah *et al.*, 2005). From all of

reviews, therefore, in this work the pCAMBIA 1302 plasmid (CAMBIA, Australia) carrying the HYG resistance genes and the GFP reporter gene controlled by a 35SCaMV promoter was used as the plasmid vector in the biolistic bombardment experiment.

Besides the reporter and selectable marker genes, polymerase chain reaction (PCR) technique has been used to screen and identify transformed plants whether containing inserted transgenes or not. Sequencing is also carried out on PCR products to verify the validity of the identity of transgenes detected.

However, study on the regeneration capacity of *EgTCTP* gene in oil palm plant is very difficult due to its long culture period. Thus new approach is tested in another monocots plant which has the shorter life cycle and easily to induce plant regeneration, *Phalaenopsis* orchid species. *Phalaenopsis*, a member in the family Orchidaceae, is one of the most popular epiphytic orchids with high economic value in the trade all over the world because of a good form, beauty, long-lasting flowers and long inflorescence suitable for ornamental plants. The regeneration pathway through culture of inflorescence stem nodes of *Phalaenopsis* is either via protocorm-like bodies (PLB) or via adventitious shoot proliferation. In general, orchid PLBs were widely used in the transformation research (Chai *et al*., 2002; Liau *et al*., 2003; Chan *et al*., 2005) because the origin of orchid PLB is a single somatic cell that why they are genetically uniform to be the complete plant. Moreover, PLB have high capability of regenerating into new plant, easy to root and can be induced efficiently from various somatic tissues including young leaves, stem segments (Sanjaya and Chan, 2007; Yee *et al*., 2008). Nowadays genetically transformed plants of a *Phalaenopsis* orchid were developed from both *Agrobacterium*-mediated (Belarmino and Mii, 2000; Mishiba *et al.*, 2005; Sreeramanan *et al.*, 2008) and particle bombardment transformation (Anzai *et al.*, 1996; Umemura *et al.*, 2007). Some genes of interest from orchid genomes or other plant species would be introduced for improving commercial traits such as fragrance enhancement. However, the goal of this experiment is to transform the *EgTCTP* gene into monocots plants both in oil palm and orchid explants to confirm the *EgTCTP* role in the monocots group.

1.3 Objective

1.3.1 To transform and regenerate oil palm plant using biolistic methods

1.3.2 To improve the plant transformation efficiency with appropriate oil palm explants for achieving stable gene transformation.

1.3.3 To validate the potential function of *EgTCTP* in oil palm as well as in other monocots such as *Phalaenopsis* orchid.

CHAPTER 2
RESEARCH METHODOLOGY

2.1 Material and Equipment

2.1.1 Plant materials used as target tissues.

2.1.1.1 Oil palm : *Elaeis guineensis* Jacq., var. *tenera*.

Mature fruits of oil palm were provided by a local oil palm company in Nakhon Si Thammarat Province, Thailand is the source of oil palm explants. Three explant types were used in this research which are zygotic mature embryo, approximately 5 mm callus in size and germinated seedling from embryo of oil palm.

2.1.1.2 The PLBs of *Phalaenopsis* orchid species obtained from Plant Biotechnology Unit, Department of Biology, Faculty of Science, Prince of Songkla University.

2.1.2 Plasmid vector

The pCAMBIA 1302 plasmid (CAMBIA, Australia), consisting of *tctp* gene as gene of interest, *hpt* gene as the selective marker and *mgfp*5 as the reporter gene under the transcriptional control of CaMV 35S constitutive promoter (Figure 4). This plasmid is approximately 10,549 bp in size (Figure 5).

2.1.3 *EgTCTP* gene used as transgene. It was cloned by Alisa Nakkaew (AAQ87663).

2.1.4 Primers

The nucleotide primers for PCR are shown in Table1.

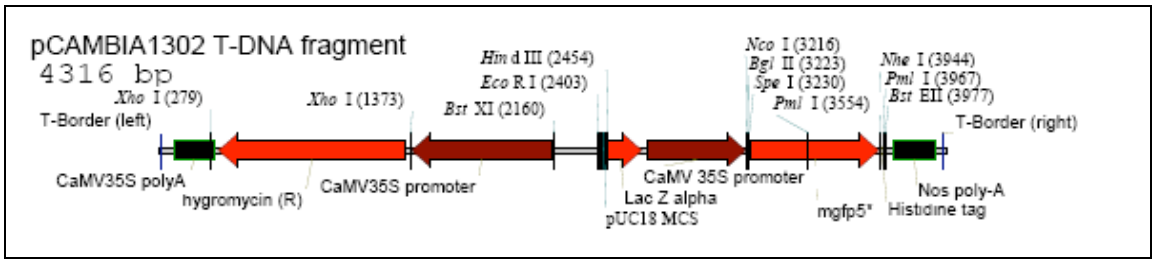


Figure 4 Schematic representation of the T-DNA of the pCAMBIA 1302 plasmid used for transformation of *E. guineensis* and *Phalaenopsis* orchid containing the *mgfp*5 and *hpt* genes under CaMV 35S promoter.

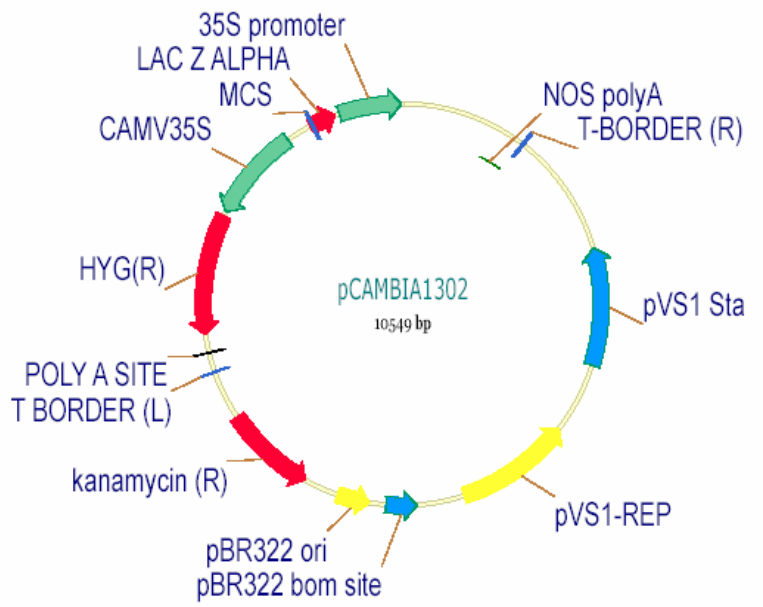


Figure 5 The map of pCAMBIA 1302 vector.

Source: www.cambia.org

Table 1. The sequences of the primer used for PCR analysis.

Primer	PCR fragment size (bp)	Sequence
5'-18S rRNA	540	5'- CAAAGCAAGCCTACGCTCTG-3'
3'-18S rRNA	540	5'- CGCTCCACCAACTAAGAACG-3'
5'- *gfp*	362	5'- TCAGTGGAGAGGGTGAAGGTGATG -3'
3'- *gfp*	362	5'- CGTTGTGGGAGTTGTAGTTGTATTC -3'
5'- *tctp-gfp*	887	5'- ATGGTAATGTTGGTTTATCAG -3'
3'- *tctp-gfp*	887	5'- AATACAACTACAACTCCCACAAC-3'

2.1.5 Equipment

The PDS-1000/He particle delivery system (BioRad, USA, Figure 6) with user supplied components: helium supply, vacuum source and consumables which contain the optimization kit such as gold microcarriers, rupture disks (ranging from 450 psi to 2,200 psi), macrocarriers and stopping screens. Macrocarrier holders and the disk-vac are also provided (Appendix A).

Figure 6 The Biolistic PDS-1000/He particle delivery system.
Source: Biolistic® PDS-1000/He Particle Delivery System Catalog

2.2 Method

2.2.1 Plant tissue culture technique

All media were solidified with 0.15% Gelrite. The concentration of sucrose was 3% (w/v) and the pH of all media was adjusted to 5.6 with 0.1 N Sodium hydroxide (NaOH) or Hydrochloric acid (HCl) prior to the addition of Gelrite. The media were autoclaved at 121°C for 20 min and dispensed 25-ml aliquots into 115-ml screw-topped jars. All experiments were carried out with 30 cultures per treatment and the experiments were repeated twice. Cultures were incubated at 26 ± 1°C with a 16-h photoperiod under an illumination of 20 $\mu molm^{-2}s^{-1}$ photosynthetic photon flux density provided by Gro-Lux light. Moreover, all cultures were sub-cultured onto fresh medium every month.

To acclimatization, germinated seedlings were transferred to the Murashige and Skoog (MS, 1962) culture medium (Appendix B) supplemented with 0.05% activated charcoal (AC) to foster root elongation for 3 weeks. Agar was carefully washed from all plants and then transferred to black plastic pots filled with potting soil until root systems were well established. Acclimatized plants were maintained in a nursery under shading, natural photoperiod and high relative humidity for 2 months and normal looking plants were obtained.

2.2.1.1 Tissue culture of oil palm

Seeds of oil palm *Elaeis guineensis* Jacq., var. *tenera* were removed from the mature fruits and used in the experiment. The expose hard endocarp was cracked open with a hammer and the kernels (zygotic embryo embedded endosperm) were surfaced sterilized by a 10-min immersion in 70% (v/v) ethanol and then cut into small cubes. These 0.5 x 0.5 cm^2 cubes were then surface sterilized by a 30-min immersion in 40% (v/v) Clorox™ solution containing 1-2 drops of Tween 20® per 100 ml of solution. Thereafter, the kernels were rinsed several times with autoclaved distilled water and kept for 24 hours prior to excision. Aseptically, zygotic embryos were removed from the kernels with a scalpel filled with a #11 blade and transferred to culture medium.

The embryos were cultured on MS medium without growth regulator for germination into seedlings while 2,4-dichlorophenoxyacetic acid (2,4-D) was added at concentration of 3 mg/l for callus induction (Patcharapisutsin and Kanchanapoom, 1996). In addition, the zygotic embryo was eliminated after the callus induction step. Small calli were maintained in the same medium until embryogenic calli were formed within 4-8 weeks and then used for transgenic studies. For transformation study, the target explants are callus, mature zygotic embryo and young seedling of oil palm.

In callus explant, growth rate of oil palm callus was calculated as the change in callus weight divided by the number of callus plated and days of culturing (mg callus^{-1} day^{-1}). In addition, growth rate of mature zygotic embryos and seedlings of oil palm were calculated as an average height (mm).

After bombardment, the transformed calli that survived from the selective medium were transferred to MS medium supplemented with 0.05% AC and one of the following growth regulators: 5 mg/l 2,4-D, 1, 3, or 5 mg/l phenyl-N^{1}-1, 2, 3,-thiadizol-5-ylurea (TDZ), 10, 15, or 20 mg/l chitosan (10 KDa shrimp oligomer chitosan kindly provided by Center for Chitin and Chitosan Biomaterials, Chulalongkorn University, Bangkok, Thailand) for plant regeneration study. Plant regeneration was recorded at fortnightly intervals for a period of 4 months. The percentages of regenerated shoot and root formation were calculated.

All experiments were conducted on three times with at least 30 replicates per treatment.

2.2.1.2 Tissue culture of *Phalaenopsis* orchid

The target tissue used in this research is PLB of *Phalaenopsis* orchid. These orchid PLBs obtained through leaf segment culture were produced by culturing in solid MS medium supplemented with 15% coconut water (CW) and maintained with four-week sub-culturing interval. Then, these PLBs were proliferated in liquid medium with shaking at 100 rounds per minute (rpm) for 2 weeks prior to transformation. All experiments were conducted on three times with at least 30 replicates per treatment.

After bombardment, the transformed PLBs growing with green coloration which survived from the selective medium were selected as putative transformants and transferred to the same medium for regeneration.

On regeneration evaluation of *Phalaenopsis* PLBs, regeneration frequency was recorded as a mean percentage of explants with shoot induction (number of regenerated explants/total number of explants x 100), time of shoot primordial initiation in days, number of regenerated shoots per explant, and scoring system was used to evaluate the regeneration efficiency as following: Negative result = 1; Below average = 2; Average = 3; Above average = 4 and Excellent = 5.

2.2.2 Construction of recombinant TCTP for plant transformation

The forward PCR primer corresponding to the beginning of the open reading frame (ORF) with the addition of an upstream in-frame *EcoR* I restriction site, (5'- GAA TTC CAT GTT GGT TTA TCA GGA TCT T-3') and the reverse primer corresponding to the 3' end of the coding region flanked by a *Bam*H I restriction site (5'- GGA TCC TTA ACA CTT GAT CTC CTT CAG C -3') were used to amplify *EgTCTP*. The PCR parameters were initially 95^{o}C for 5 min followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min and then a final extension for 10 min was performed at 72°C. The PCR products were ligated to a similarly digested pCAMBIA 1302 carrying the GFP reporter gene controlled by a CaMV35S promoter. The inserted DNA fragments were sequenced using the ABI prism 377 apparatus to ensure the authenticity of the cloned nucleotide sequence.

2.2.3 Microprojectile bombardment experiment

Bombardment experiments were performed according to the manufacturer's instructions for the Biolistic PDS-1000/He particle delivery system and all the bombardments were performed according to the standard procedure. Each petri dish was bombarded once but each parameter was performed in triplicates.

2.2.3.1 Preparation before performing a bombardment

One day prior to transformation, all kinds of target explants were placed in a circle with a diameter of 25 mm at the centre of a 90-mm diameter petri dish on fresh solid medium for the highest frequency of stable transformants in bombardment experiment as described by Parveez, *et al*. (1997). Three controls were included, which consisted of unbombardment explants, explants bombarded with microcarriers only and explants bombarded with microcarriers coated with plasmid vector (not coated with DNA).

- Pre-assemble and pre-sterilize consumable materials

All materials such as the macrocarriers (Figure 7A), macrocarrier holders, rupture disks (Figure 7B) and stopping screens (Figure 7C) were pre-assemble and pre-sterilize by autoclaving except rupture disks sterilized by briefly dipping them in 70% isopropanol just prior to insertion in the retaining cap. Especially for microcarriers, the procedure prepares tungsten (Figure 7D) or gold microcarriers (Figure 7E) based on the method of Sanford, *et al.* (1987).

For 120 bombardments using 500 µg of the microcarrier, each preparation 30 mg of microparticles were weighted out into 1.5 ml microfuge tube, 1 ml of 70 % (v/v) ethanol was added to it. The microfuge tube was vortexed vigorously for 3-5 minutes and then, the particles were remained soaked in 70 % (v/v) ethanol for 15 minutes. The microparticles were pelleted by centrifugation for 5 seconds at 14,000 rpm. The microparticles were washed three times by adding 1 ml of sterile water, vortexing vigorously for 1 minute allowing the particles to settle for 1 minute and pelleting the microparticles by briefly centrifugation. After the third wash, the particles were suspend in 500 µl of sterile 50% (v/v) glycerol and stored at -20 ºC at a final concentration of 60 mg/ml.

- Coating washed microcarriers with DNA

The following process is sufficient for 6 bombardments; if fewer bombardments are needed, adjust the quantities accordingly.

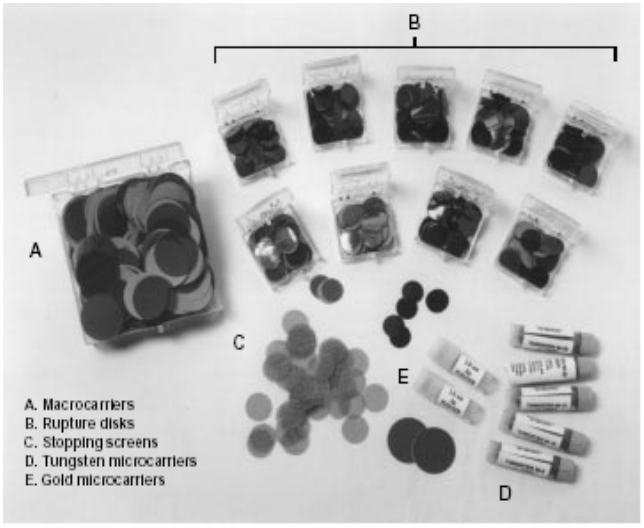


Figure 7 Consumables for the PDS-1000/He instrument.
(A) macrocarriers, (B) rupture disks (C) stopping screens
(D) tungsten microcarriers (E) gold microcarriers
Source: Biolistic® PDS-1000/He Particle Delivery System Catalog

1) Vortex the microcarriers prepared in 50% glycerol (30 mg/ml) for 5 minutes on a platform vortexer to resuspend and disrupt agglomerated particles.
2) Remove 50 µl (3 mg) of the microcarriers to a 1.5 ml centrifuge tube. While vortexing vigorously, add in order: 5 µl DNA (1mg/ml), 50 µl 2.5 M Calcium chloride ($CaCl_2$) and 20 µl 0.1 M spermidine (free base, tissue culture grade)
3) Continue vortexing for 2-3 minutes.
4) Allow the microcarriers to settle for 1 minute.
5) Pellet microcarriers by spinning for 2 seconds in a microfuge.
6) Remove the liquid and discard.
7) Add 140 µl of 70% ethanol (HPLC of spectrophotometric grade).
8) Remove the liquid and discard.
9) Add 140 µl of 100% ethanol.
10) Remove the liquid and discard.
11) Add 48 µl of 100% ethanol.

12) Gently resuspend the pellet by tapping the side of the tube several times, and then by vortexing at low speed for 2-3 seconds.

2.2.3.2 Performing a bombardment

1) Adjust bombardment parameters for gap distance between rupture disk to macrocarrier distance set at 11 mm, macrocarrier to stopping plate set at 11 mm and stopping plate to target tissue set at 90 mm following the protocol of Parveez *et al.* (1997; 1998). Placement of stopping screen support in proper position inside fixed nest of microcarier launch assembly (Figure 8).
2) Check helium supply (200 psi in excess of desired rupture pressure). Helium pressure of 850, 1100, 1300 and 1550 psi bombardment pressures were tested in oil palm study but only 1100 psi (Yee *et al.*, 2008) were used in orchid transformation. Treatments were replicated three times. Evaluation of helium pressure and type of explants were based on growth rate of explants.
3) Load 1.0 μm diameter gold microcarriers coated DNA onto sterile macrocarrier or macrocarrier holder.
4) Plug in power cord form main unit to electrical outlet and power ON.
5) Sterilize chamber walls with 70% ethanol.
6) Load sterile rupture disk into sterile retaining cap.
7) Secure retaining cap to end of gas acceleration tube (inside, top of bombardment chamber) and tighten with torque wrench.
8) Load macrocarrier and stopping screen into microcarrier launch assembly.
9) Place microcarrier launch assembly and target cell in chamber and close door.
10) Evacuate chamber, hold vacuum at 28 inches of mercury (Hg). This vacuum level is useful for most plant cells or tissues.
11) Bombard sample by depressing the Fire button continuously until rupture disk bursts and helium pressure drops to zero
12) Release Fire button.

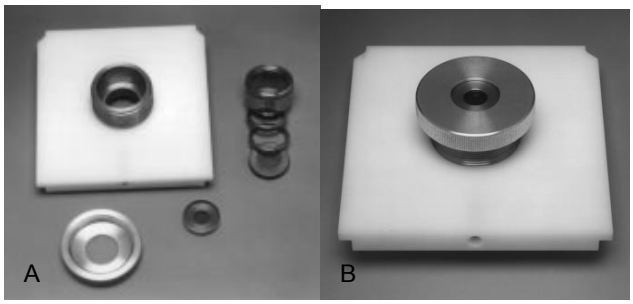


Figure 8 Microcarrier launch disassembly (A) and assembled components (B).

Source: Biolistic® PDS-1000/He Particle Delivery System Catalog

2.2.3.3 After performing a bombardment

1) Release vacuum and remove target cells from chamber.
2) Unload macrocarrier and stopping screen from macrocarrier launch assembly.
3) Unload spent rupture disk.
4) Remove helium pressure from the system (after all experiments completed for the day).

2.2.4 Analysis of transformants

2.2.4.1 Selection of putative transformants

After bombardment, all kinds of oil palm explants: embryo, callus, seedling and orchid PLBs were cultured on fresh MS medium for one week to recovery from the injured bombardment. Then, for selection of transformants, all transformed explants were transferred into selective medium which have been determined previously. HYG at a final concentration of 50 μg/ml (Abdullah *et al.,* 2005) and 20 μg/ml (Mishiba *et al.*, 2005) was used in the maintenance medium for oil palm and orchid selection, respectively. The explants were subcultured to fresh selective medium at monthly intervals for 2 months.

The transformed explants of oil palm survived after selection in MS medium containing 50 μg/ml HYG and the untransformed explants which cultured on the normal MS medium were investigated and compared in growth rate after bombardment until 6 months. Except the oil palm seedling, the observation was still continue done after transferred to the greenhouse after bombardment until 1 year and 6 months. Whereas, the escaped PLBs of *Phalaenopsis* orchid from the selective medium were compared in regeneration evaluation after bombardment until 6 months.

Data were recorded based on the percentage of surviving explants (number of surviving explants/total number of explants x 100) and also used as transformation efficiency.

2.2.4.2 GFP detection

A small section of surviving explants of oil palm and orchid were observed for their GFP expression with a confocal laser scanning microscope equipped with GFP filter set for excitation between 455 and 490 nm and emission above 500 nm. The expression of the *gfp* gene was determined as bright-green fluorescence caused by GFP accumulation in the cells. Importantly, the difficulty of detecting GFP in autofluorescence of the chlorophyll as in the leaf of oil palm seedling and PLBs of orchid was overcome by using triple colour images which acquired by sequentially scanning with settings optimal for *gfp* (green), autofluorescence of the chlorophyll (red) and surface of organelle (blue). Filter sets tailored to the specific chromophores were used for GFP (excitation 488 nm argon laser line and emission collected from 500 to 543 nm, channel 1); autofluorescence of the chlorophyll (excitation 543 nm argon laser line and emission collected from 610 to 630 nm, channel 2) and surface of organelle (excitation 650 nm argon laser line and emission collected from 667 to 750 nm, channel 3). Reflected light images were obtained by detection of light at the excitation wavelength. Serial confocal optical sections were taken at different step sizes.

The images of the individual channels were merged and stored as TIF files to facilitate visualization. Monitoring was carried out at random 3 days, 1, 2, 3, 4 weeks, 2, 3, 4, 5 and 6 months after bombardment.

2.2.4.3 Histological analysis

Light microscopy samples were fixed in formaldehyde–acetic acid–alcohol (FAA) fixative (85:5:10; 100% acetic acid: 100% formaldehyde: 96% ethanol). After fixation, the samples were run in an alcohol series for dehydration starting with 70% ethanol and ending up with absolute ethanol. After dehydration, the light microscope samples were embedded in paraplast and cut in sections (ca. 10 μM). The tissue slices were placed on glass, stained with haematoxylin and safranin for 5 minutes and rinsed with distilled water. All sections were mounted with permount and viewed under bright-field illumination with an Olympus microscope. The samples were analysed as the average cell density per area (mm^2) from 10 replicates in each explants type and photographed.

2.2.4.4 DNA isolation and molecular analysis for detection of transformants

After obtaining the transformants, surviving seedlings and viable embryogenic calli of oil palm and the *Phalaenopsis* PLBs were selected and subsequently transferred to regeneration media. Moreover, to verify for the presence of the transgenes and confirm the stable integration of the DNA into the plant genome, the putative transformants were analyzed by PCR using two pairs of primers; GFP forward / GFP reverse primers and EgTCTP:GFP forward / EgTCTP:GFP reverse primers which visualized by ethidium bromide staining after agarose gel electrophoresis.

- Genomic DNA isolation

According to manufacturer's instructions of Genomic DNA extraction Kit (Plant) Mini (RBC Bioscience Corp., Taiwan), total genomic DNA were extracted from the oil palm explants and leaves of

regenerated bombarded *Phalaenopsis* plantlets which analyzed for the presence of the *gfp* and *tctp-gfp* genes. Briefly, 100 mg of plant tissues were cut and grinded with a homogenized blue pestle to a fine powder in the first step. The sample is lysed by GP1 (or GPX1) buffer and then the lysate is treated with RNase A to remove RNA. Add GP2 buffer and mix by vortexing. Incubate on ice and add GP3 buffer. Apply the entire lysate from previous step to the filter column. In the presence of a chaotropic salt, the genomic DNA in the lysate binds to the glass fiber matrix in the spin column. The contaminants are washed with an ethanol based wash buffer and purified genomic DNA is eluted by low salt elution buffer or distilled water (Figure 9). The genomic DNA was electrophoresed in a 0.8% agarose gel. Lambda DNA HindIII Markers were used as a size standard for fragments larger than 1 kb. Gel was stained with Ethidium Bromide to view fragments.

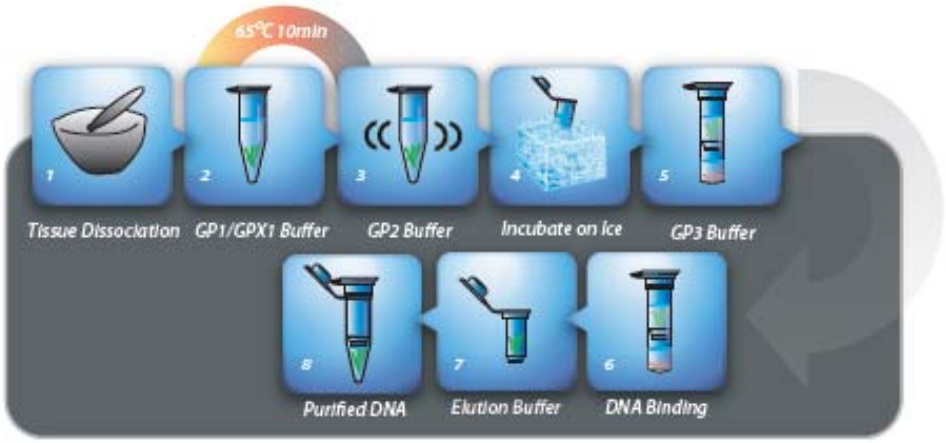


Figure 9 Eight steps of genomic DNA extraction Mini Kit (Plant).

Source: www. rbcbioscience.com

- PCR analysis

Genomic DNA was extracted from about 100 mg of putative transgenic plants using a Genomic DNA extraction Mini Kit (Plant). To confirm the presence of the *gfp* and *tctp-gfp* genes in the transgenic plants, PCR experiments were performed using specific primers for GFP and EgTCTP:GFP. For the first PCR primer pair, it was designed to amplify an

internal 362 bp *gfp* fragment: *gfp*F, 5'TCAGTGGAGAGGGTGAAGGTGA-TG3' and *gfp*R 5'CGTTGTGGGAGTTGTAGTTGTATTC3'. For another PCR primer pair, resulting in a 887 bp fragment of *tctp-gfp* genes, *tctp-gfp*F: 5'ATGGTAATGTTGGTTTATCAG3' and *gfp*R 5'CGTTGTGGGAGTTGT-AGTTGTATTC3' were used in the PCR reaction.

In the PCR reactions, amplifications of the *gfp* and *tctp-gfp* genes each were carried out in a 12.5 µl reaction volume containing 1.25 µl of 10 x PCR buffer (10 mM Tris-Cl, pH 8.8, 50 mM KCl and 0.8% Nonidet P40), 0.75 µl of 1.5 mM MgCl2, 0.25 µl of 5 mM dNTP mix (2.5 mM of each nucleotide dATP,dCTP, dGTP, and dTTP), 0.1 µl of 5 U Taq polymerase, 0.25 µl of 15 pmole primer each, 1 µl of template DNA at 100 ng/µl and 8.65 µl nuclease-free water.

The PCR run condition was set to an initial denaturation step of 5 minutes at 94°C and subsequent 35 cycles of denaturation (94°C, 50 seconds), annealing (50 °C, 1 minute) and extending (72°C, 1 minute) followed by a final extending step at 72°C for 10 min. The PCR products were electrophoresed in a 1.5% agarose gel. The size of the amplified fragments were determined with reference to 100 basepair DNA ladder Gel was stained with Ethidium Bromide to view fragments.

2.2.5 Data analysis

All experiments were conducted on three different days with at least 30 replicates per treatment. Population size varied between experiments, and the size is indicated together with the experiment results. Analysis of variance (ANOVA) was applied to the results wherever indicated using Sheffe's test at $P \leq 0.05$. The software used was SPSS 13.0 (SPSS Inc., USA) for Windows XP Professional.

CHAPTER 3
RESULTS

3.1 Chitosan effect on oil palm regeneration experiment

Attempts were made to initiate plantlet production through the application of 2, 4-D, TDZ and chitosan either singly or in combination. The surviving calli obtained from biolistic experiment were transferred to these media. Only yellow compact calli (Figure 10A) obtained from zygotic embryos showed distinct morphological responses and the fast growing calli were obtained. The newly formed callus was granular and could be separated from each other easily. Shoot-like growth without roots emerged on MS medium containing 15 mg/l chitosan either with or without 5 mg/l 2, 4-D (Figure 10B). Thus, at the multiple shoot stage, each shoot was excised and transferred to the same medium in the presence of 0.05% AC for root induction for 3 weeks. It is interesting to note that prolonged culture on this medium allowed the regenerated plants to produce *in vitro* flower (Figure 10C). All seedlings with well-developed shoot and root were transferred to small black plastic pot containing sterile vermiculite. These seedlings were covered with a transparent plastic sheet and watered with medium lacking sucrose: distilled water 3: 1 for 4 weeks. They subsequently continued to grow fairly uniform plants (Figure 10D).

From table 2, the acquisition of organogenic competence was influenced by the presence of chitosan either alone at the concentrations of 15 and 20 mg/l or combined with 5 mg/l 2, 4-D shoot formation was evidenced. The highest percentage of shoot formation was 16% at 15 mg/l chitosan while 12% of shoot formation was observed at 15 mg/l chitosan in combination with 5 mg/l 2, 4-D. No root was formed with the application of chitosan solely but root formation was evidenced in the media containing combinations of 2, 4-D and TDZ or 2, 4-D and chitosan.

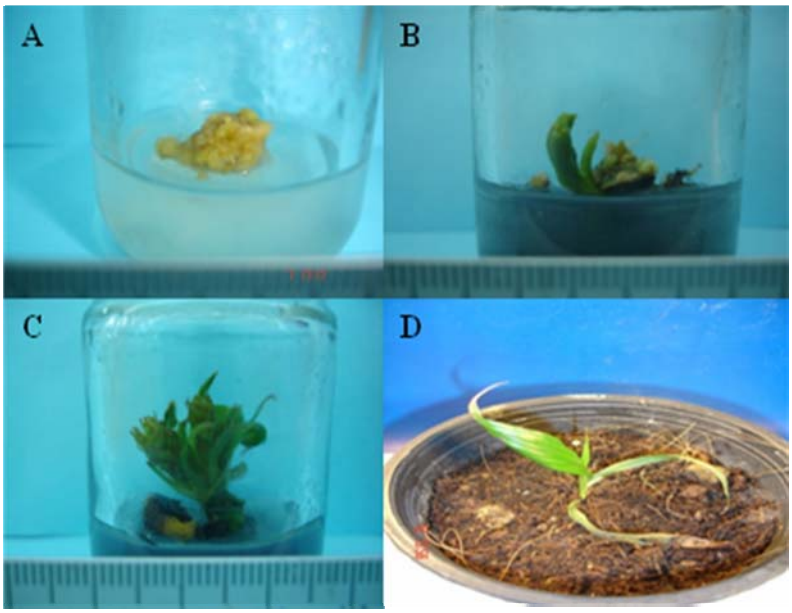


Figure10 Organogenesis in oil palm.

(A) Yellow compact callus derived from embryo.

(B) Regeneration of plant derived from yellow compact callus cultured on MS medium containing 15 mg/l chitosan.

(C) Development of *in vitro* inflorescence.

(D) Plantlet obtained from organogenesis in potting soil.

3.2 Evaluation of helium pressure and type of oil palm explants using in biolistic gene transfer

As used herein, the term "**control**" refers to the unbombarded explants and the transformed explants bombarded with only gold particle, "**vector**" refers to the transformed explants bombarded with gold particle and pCAMBIA 1302 vector and "**gene**" refers to the transformed explants bombarded with gold particle and the *EgTCTP*-pCAMBIA 1302 vector.

Zygotic calli and seedlings of oil palm, 1 day after subculture, or freshly mature embryo culture, were bombarded with plasmid pCAMBIA1302-*EgTCTP* DNA. However, unbombarded explants and bombarded without plasmid DNA and without pCAMBIA1302 vector were included in all experiments as a control. After being bombarded and kept on callus maintenance medium for 3 days, the cells were transferred to callus maintenance medium containing 50 mg/l HYG. Eight weeks after

Table2 *In vitro* response of granular callus of oil palm cultured on MS medium supplemented with different concentrations of 2,4-D, TDZ and chitosan.

Plant growth regulators (mg/l)			Shoot formation	Root formation
2,4-D	TDZ	Chitosan	(%)	(%)
0	0	0	0^{c}	0^{e}
0	1	0	0^{c}	0^{e}
0	3	0	0^{c}	0^{e}
0	5	0	0^{c}	0^{e}
0	0	10	0^{c}	0^{e}
0	0	15	16^{a}	0^{e}
0	0	20	3^{c}	0^{e}
5	1	0	0^{c}	75^{a}
5	3	0	0^{c}	60^{b}
5	5	0	0^{c}	50^{c}
5	0	10	0^{c}	50^{c}
5	0	15	12^{b}	35^{d}
5	0	20	2^{c}	30^{d}

Each value represents the mean of fifteen replicates. Means within a column followed by different letters show significant difference as analyzed by Sheffe's test at $p \leq 0.05$.

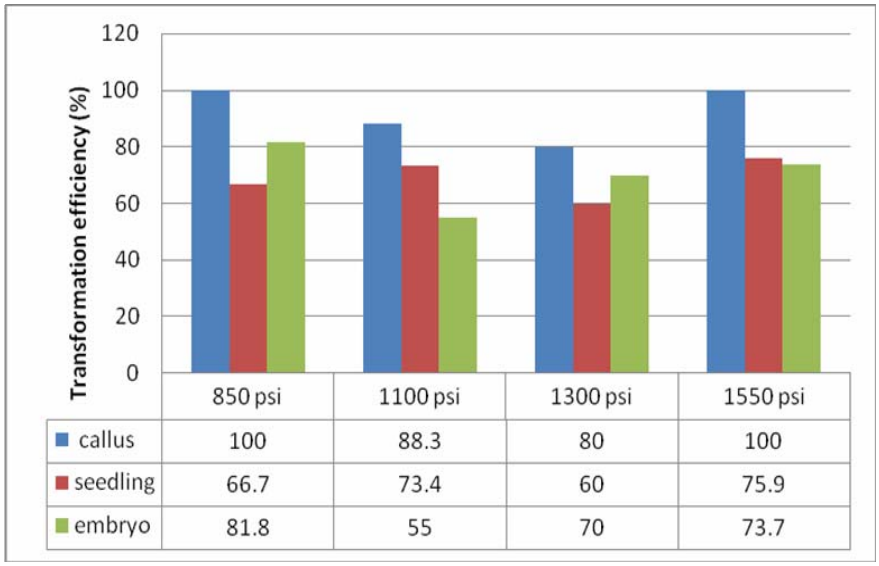
selection, HYG-resistant explants continued to grow and looked healthy as normal whereas no resistant explants were observed on control plates in any experiment. From observation, the controlled explants without pCAMBIA 1302 vector became black and died within 1 month after bombardment. Consequently, the percentages of survival explants were calculated in 2 months after subculturing into the HYG culture medium.

The transformation efficiencies obtained with mature embryos, zygotic calli and seedlings as target tissues were compared at 3 months post bombardment (Figure 11). In only 2 sets of experiments, we obtained a 100% transformation efficiency but the average total percentage of resistant explants was closer to 77%. The complete 100% survival of HYG resistant explants was obtained in the bombarded callus using 850 and 1550 psi helium pressure. However, the transformation efficiency using helium pressures of 1100 and 1300 psi for callus bombardment were high but limited only 80.0-88.3%. For seedlings, transformation efficiency was over a limited range of 60.0 to 75.9% at the different helium pressures with the highest number of surviving seedlings recovered after bombardment at 1,550 psi. For embryos, the transformation frequency was over the range of 55.0 to 81.8% with the highest viability of embryos after bombardment at 850 psi. The average total of resistant explants from three separate experiments was 92% for callus, 69% for seedlings and 70% for embryos. The resistant calli were subsequently transferred to fresh plant regeneration medium.

3.3 Growth rate analysis of oil palm after biolistic transformation

3.3.1 Growth rate analysis of oil palm callus

To observe this experiment, the calli were weighed and calculated as the growth rate data. At the optimal helium pressure level for callus transformation, 1100 psi, the *EgTCTP* gene transformed callus gave highly significant differences in callus growth rate (Figure 12) at P = 0.05 level which was 0.51 mg per callus per day. Meanwhile the growth rate of control and vector transformed groups were only 0.03 and 0.02 mg per callus per day, respectively. Anyway, no significantly different between groups of oil palm callus in the three residual helium pressures at 850, 1300 and 1550 psi were found (Figure 13).



	850 psi	1100 psi	1300 psi	1550 psi
callus	100	88.3	80	100
seedling	66.7	73.4	60	75.9
embryo	81.8	55	70	73.7

Figure 11 Comparison of transformation efficiency (%) at 3 month after bombardment in mature embryo, zygotic callus and seedling as target tissues used to produce transgenic oil palm plants (each experiment was 3 replicates performed, n=30).

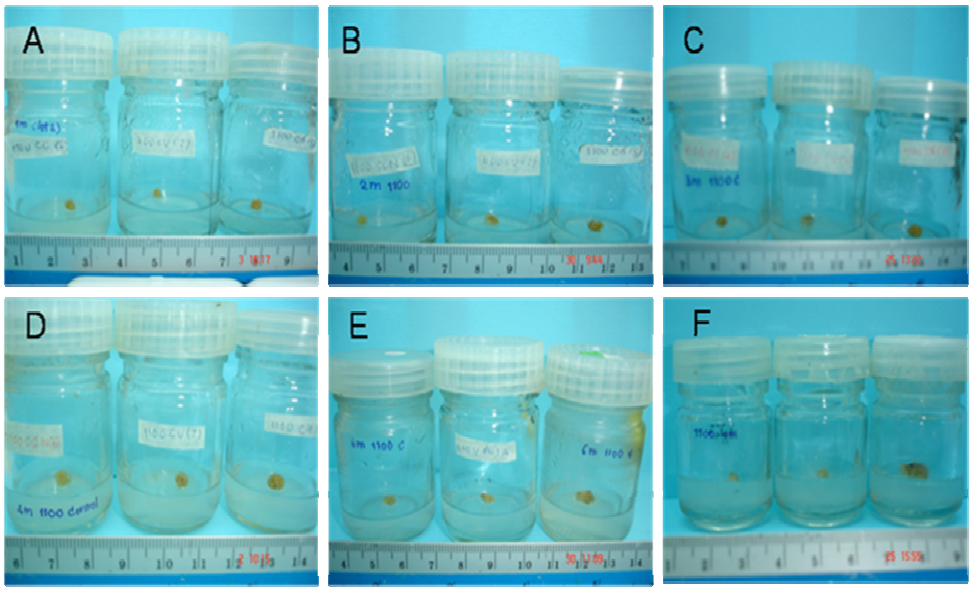


Figure12 Callus of oil palm 1-6 month (A-F, respectively) post-bombardment at helium pressure 1100 psi. From left to right in each picture is the control, vector-transformed and *EgTCTP* gene transformed callus was compared in growth rate.

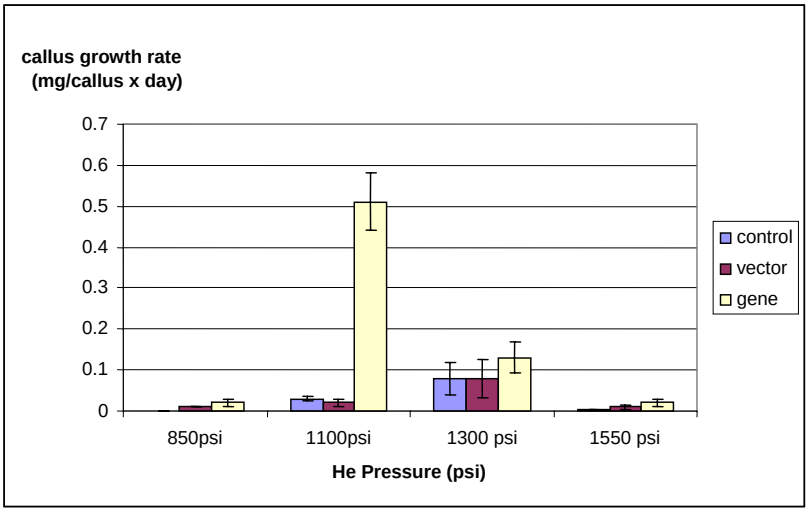


Figure 13 Comparison of growth rate in oil palm callus at 6 months after transformed the *EgTCTP* gene via biolistic method with four levels of helium pressure (n= 30). Bars represent standard error.

3.3.2 Growth rate analysis of oil palm seedling

All oil palm seedlings were measured and calculated as the growth rate data. For the first six months after bombardment period, the seedlings at four levels of helium pressure (850-1550 psi) were cultured in the sterile condition. The height of oil palm seedlings in each helium pressure is not different between control, vector and gene at every bombarded pressure (Figure 14). In this period, no significantly different between groups of oil palm seedlings were found in all level of the helium pressures at 850, 1100, 1300 and 1550 psi (Figure 15).

After that all seedlings were acclimatized to vermiculite (Figure 16A) and then transferred to the pot in the greenhouse (Figure 16B-E). Pinnate mature leaf was found in 1 year and 3 months after bombardment (Figure 16D-E). These oil palm seedlings were observed monthly for 1 year and six months after bombardment. Unfortunately, only 13 seedlings (that is 16.2% of all transformants) of oil palm (Figure 16C) could survive until now except the entire vector transformed seedling bombarded at 1550 psi helium pressure died. The appearance of oil palm seedlings at 1 year and 6 months after transformation when bombarded at 1100, 1300 and 1550 psi without DNA, with pCAMBIA1302 vector and with *EgTCTP* gene are shown in Figure 17.

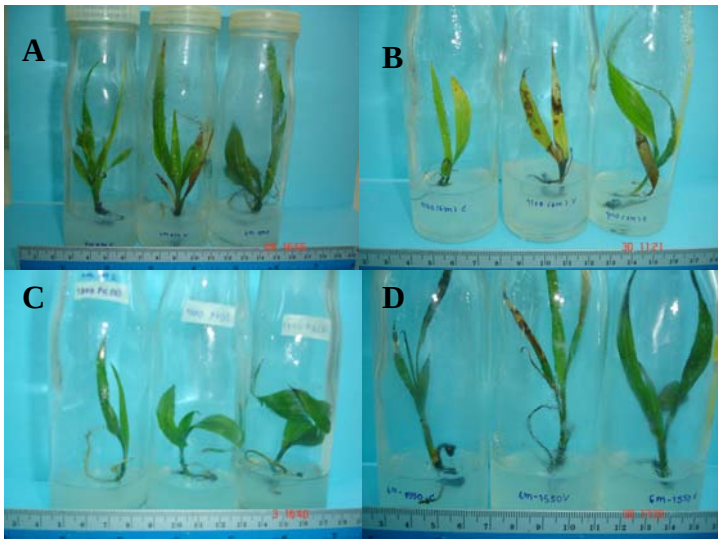


Figure14 Effect of different helium pressure (psi) on the oil palm seedlings growth 6 months after transformation.

(A) From left to right, bombarded seedling at 850 psi without DNA, with pCAMBIA1302 vector and with *EgTCTP* gene,

(B) From left to right, bombarded seedling at 1100 psi without DNA, with pCAMBIA1302 vector and with *EgTCTP* gene,

(C) From left to right, bombarded seedling at 1300 psi without DNA, with pCAMBIA1302 vector and with *EgTCTP* gene and

(D) From left to right, bombarded seedling at 1550 psi without DNA and with *EgTCTP* gene.

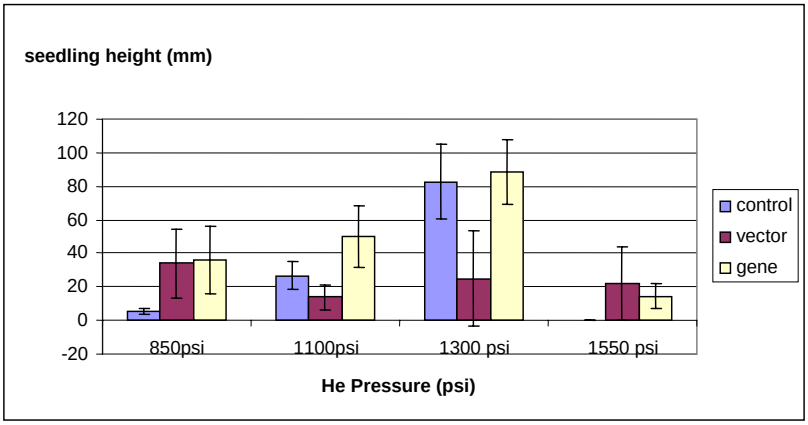


Figure 15 Comparison of seedling height (mm) in oil palm seedling at 6 months after transformed the *EgTCTP* gene via biolistic method with 4 levels of helium pressure (n =30). Bars represent standard error.

Figure16 Acclimatization of oil palm seedlings to vermiculite (A); then transferred to small pots (B) and the bigger ones when grew up which placed in the closed green house(C-E). Mature leaf, pinnate type (D-E), was found in 1 year and 3 months after bombardment.

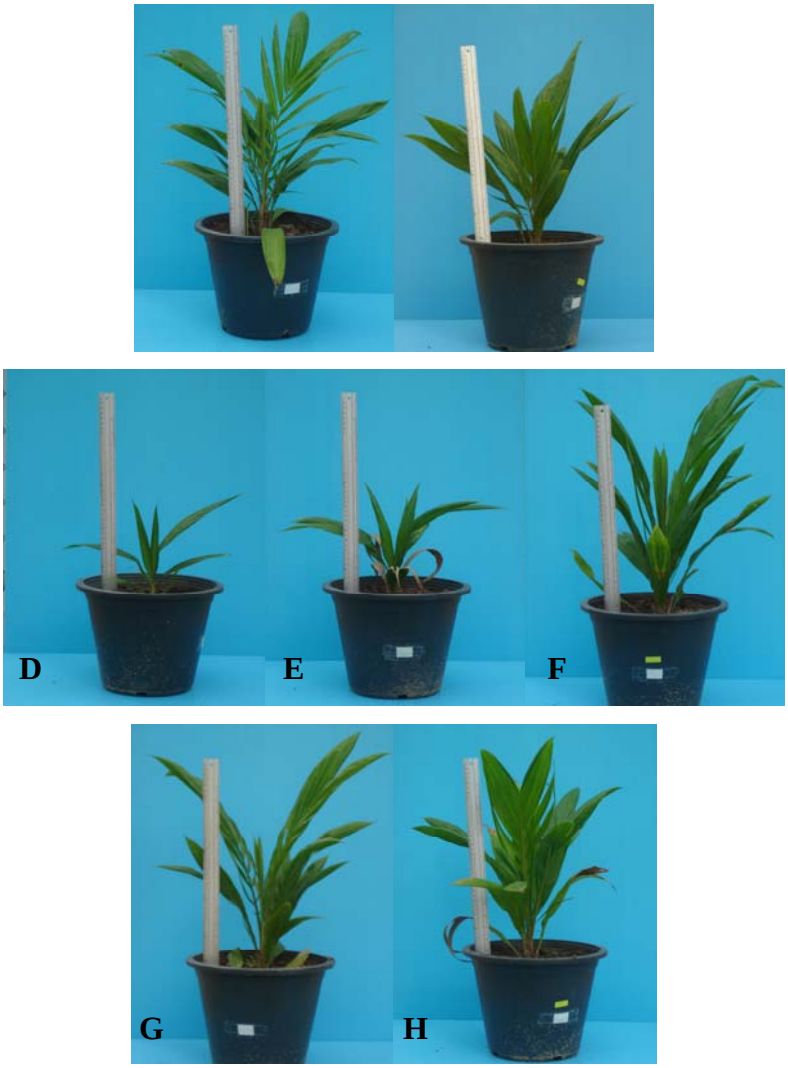


Figure17 Effect of different helium pressure (psi) on the oil palm seedlings growth 1 year and 6 months after transformation.
(A) Bombarded seedling at 1100 psi without DNA,
(B) Bombarded seedling at 1100 psi with empty pCAMBIA1302 vector,
(C) Bombarded seedling at 1100 psi with *EgTCTP* gene,
(D) Bombarded seedling at 1300 psi without DNA,
(E) Bombarded seedling at 1300 psi with pCAMBIA1302 vector,
(F) Bombarded seedling at 1300 psi with *EgTCTP*,
(G) Bombarded seedling at 1550 psi without DNA and
(H)Bombarded seedling at 1550 psi with *EgTCTP* gene.

However, the residual oil palm seedling bombarded with the *EgTCTP* gene at 1300 psi gave highly significant differences in growth rate at P = 0.05 level which was 570 mm when the samples were monitored in the next 1 year period. At 850 psi helium pressure, no escape transformants were found for data analysis. At 1100 psi, the average height of germinated seedlings in control, vector alone and *EgTCTP* gene transformants were 51.5, 46.8, 43.3 cm and the average height of germinated seedlings at 1550 psi in control was 55 cm and in *EgTCTP* gene transformants was 51.3 cm (Figure 18).

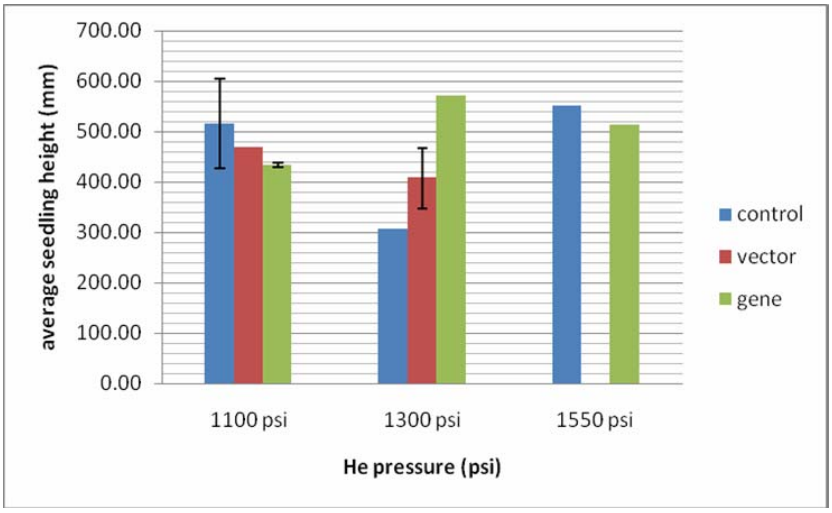


Figure 18 Comparison of average seedling height (mm) in oil palm seedling 1 year and 6 months after transformed the *EgTCTP* gene via biolistic method. From left to right, at 1100 psi helium pressure n=3, n=1 and n=2, respectively; at 1300 psi helium pressure n=1, n=3 and n=1, respectively and at 1550 psi helium pressure n=1, n=0 and n=1, respectively. Bars represent standard error.

3.2.3 Growth rate analysis of oil palm embryo

On oil palm embryo bombardment, the growth rate of mature embryo was observed as the height of new germinated seedling per embryo per day. From Figure 19, only embryos which are vector transformants germinated into the oil palm seedlings wheareas no germination was found in the control and gene

transformants of oil palm embryos. In addition, no escaped embryo was found at the helium pressure of 850 and 1300 psi at 6 months after biolistic transformation.

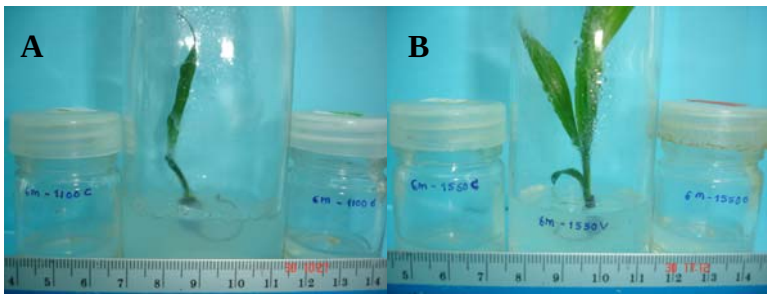


Figure19 Germination and growth of oil palm mature embryos at 6 month after bombardments at 1100 psi (A), and 1550 psi (B) level were compared. From left to right in each picture are control, vector-alone and the *EgTCTP* gene transformants.

Furthermore, the data from Figure 20 revealed that the average height of germinated seedlings at 1100 psi in control, vector alone and *EgTCTP* gene transformants were 9.9, 12 and 3 mm, respectively. At this pressure level, the transformants with the *EgTCTP* gene gave highly significant differences in growth rate at P = 0.05 level. In contrast, embryo bombardments gave no significantly different growth rate results at 850, 1300 and 1550 psi helium pressure level of bombardments. At 850 psi, the average height of germinated seedlings in control, vector alone and *EgTCTP* gene transformants were 7.6, 1.9 and 1.4 mm, respectively. At 1300 psi, the average height of germinated seedlings in control, alone vector and *EgTCTP* gene transformants were 6.8, 2.5, 5 mm and at the last helium pressure, 1550 psi, the average height of germinated seedlings in control, alone vector and *EgTCTP* gene transformants were 0, 13 and 4.4 mm, respectively.

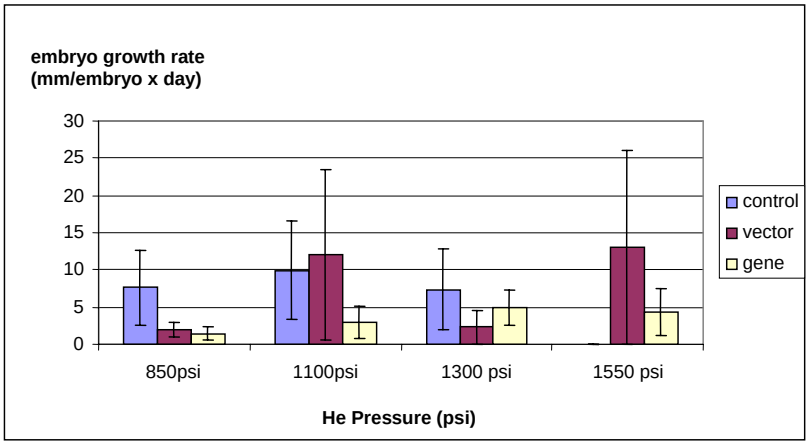


Figure 20 Comparison of growth rate in oil palm embryo 6 months after transformed the *EgTCTP* gene via biolistic method with four levels of helium pressure (n=30). Bars represent standard error.

3.4 Regeneration evaluation of *Phalaenopsis* PLBs after bombardment

Germination and growth of the *Phalaenopsis* PLBs at 1-6 months after bombardment at 1100 psi helium level were compared as illustrated in Figure 21. In the first month after bombardment, PLBs of *Phalaenopsis* which transformed *EgTCTP* gene showed the swollen differentiated tissue while the others were still not changed (Figure 21A). One month later, the *EgTCTP* transformant regenerated and formed the shoot primordial (Figure 21B). However, in the third month after biolistic transformation, the control and vector alone transformants began to regenerate (Figure 21C) and continue growing (Figure 21D).

To evaluate the regeneration of *Phalaenopsis* PLBs which bombarded at 1100 psi helium pressure, four criteria were studied as following: regeneration frequency, time of shoot primordial initiation, number of regenerated shoots per explant and regeneration efficiency. Results were summarized in Table 3.

For the first criteria, the regeneration frequency of control, vector and gene groups in *Phalaenopsis* PLBs was 78.34, 80 and 95.66, respectively. Meanwhile the gene transformants took fewer days on shoot primordial initiation than the control and vector transformants. Shoot primordial initiation time in the *EgTCTP* gene transformants took only 55.22 ± 26.56 days with 3.78 ± 1.89 of re-generated shoots per explant. Moreover, the *EgTCTP* gene transformants achieved

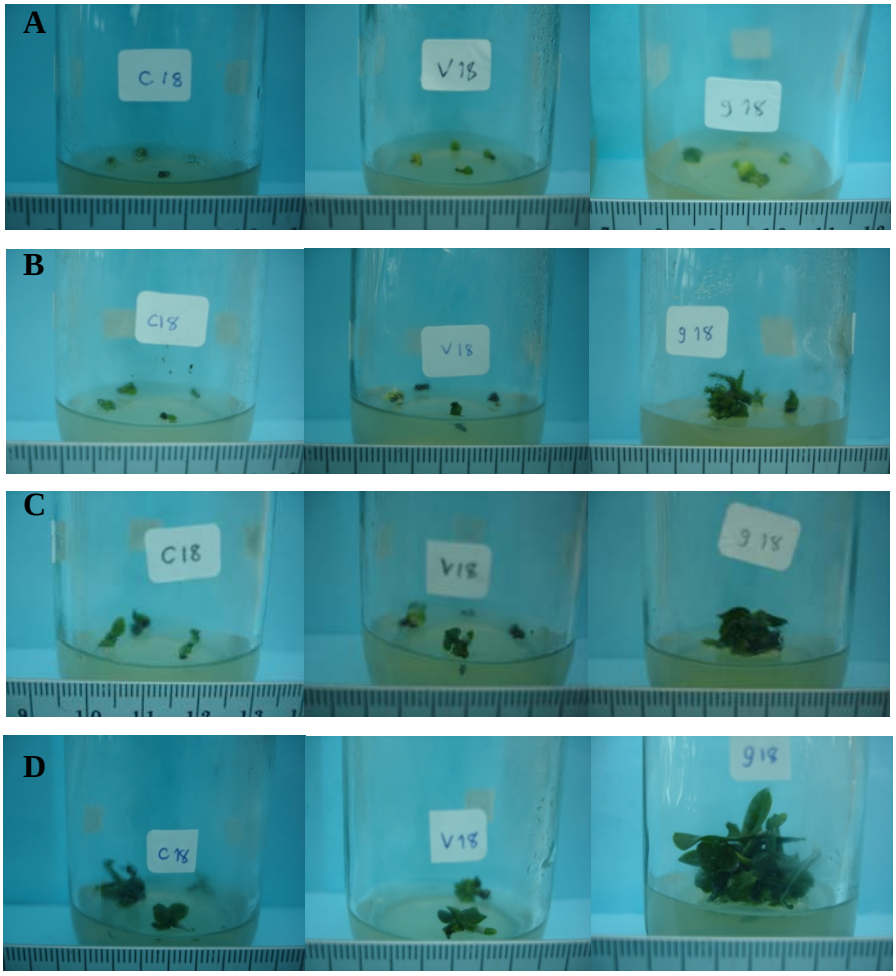


Figure 21 Germination and growth of *Phalaenopsis* PLBs 1-6 month after bombardment at 1100 psi helium level were compared. From left to right in each picture is control, vector-transformed and the *EgTCTP* gene transformed PLBs.

(A) *Phalaenopsis* PLBs 1 month after transformation,

(B) *Phalaenopsis* PLBs 2 months after transformation,

(C) *Phalaenopsis* PLBs 3 months after transformation and

(D) *Phalaenopsis* PLBs 6 months after transformation

Table 3 Regeneration evaluation parameters of regenerated *Phalaenopsis* PLBs compared in control, vector and gene groups. Each value represents the mean of thirty replicates. Means within a row followed by different letters show significant difference as analyzed by Sheffe's test at $p \leq 0.05$.

Explant group / **Evaluation criteria**	**Control**	**Vector**	**Gene**
Regeneration frequency (%)	78.34	80	95.66
Time of shoot primordial initiation (days)	83.50 ± 33.89^{a}	61.87 ± 18.52^{b}	55.22 ± 26.56^{b}
Number of regenerated shoots per explant	1.56 ± 0.51^{a}	1.14 ± 0.46^{a}	3.78 ± 1.89^{b}
Regeneration efficiency*	2.94 ± 0.52	3.16 ± 0.76	4.04 ± 0.88

* Regeneration efficiency was expressed as a score from 1 to 5 (Negative result = 1; Below average = 2; Average = 3; Above average = 4 and Excellent = 5).

the highest score of regeneration efficiency which was 4.04 ± 0.88 showing "above average" status.

With regard to shoot primordial initiation time, the control and vector transformant groups took 83.50 ± 33.89 and 61.87 ± 18.52 days, respectively. These groups took more days for primordial initiation than the gene transformed group.

In addition, numbers of regenerated shoots per explant in the control and vector transformant groups were 1.56 ± 0.51 and 1.14 ± 0.46, respectively.

For the last criteria on regeneration efficiency as scoring system, two groups of control and vector transformants could be classified into the same level. The score of control and vector group were 2.94 ± 0.52 and 3.16 ± 0.76, respectively which evaluated as the "average" status.

3.5 Visualization of GFP expression

The successful gene transformation and expression in transformants was examined with a confocal laser-scanning microscope fitted with GFP filter set. GFP gene expression was seen as a bright-green fluorescence caused by GFP accumulation in the cells compared to the control. In this research, GFP-derived fluorescence was observed in calli, embryos, leaves and roots of oil palm (Figure 22) and PLBs of *Phalaenopsis* orchid (Figure 23). Monitoring was carried out at random on bombarded explants at 1 to 6 months after bombardment. Transformants expressed bright-green fluorescence with the *mgfp5* construct. High levels of GFP were therefore expressed in all transformants when monitored 3 days (Figure 22 E-H), 1 month (Figure 22 I-L) and 6 months (Figure 22 M-P) after biolistic transformation. Nevertheless, none of the control explants fluoresced with the green spots except in the oil palm leaf (Figure 22 A-D) and the orchid PLBs which probably due to the auto-fluorescent from chloroplasts. To solve this problem, three different fluorescent, green, red and blue were scanned at the same time and overlay three filters together (Figure 23). In addition, GFP expression at 60 days after bombardment in PLBs of *Phalaenopsis* transformants was illustrated in Figure 24. When the control PLBs (Figure 24 A-B) were compared with the pCAMBIA 1302 transformants (Figure 24 C) and the *EgTCTP* orchid transformants (Figure 24 D), m*gfp5* bright green spots were seen both in the vector alone transformants and the successful gene transformants but none in the control.

3.6 Histological analysis

The histological events during callus proliferation in oil palm have been examined. Cell divisions were more frequent in the *EgTCTP* transformant than the control and vector calli as shown in Figure 25. Moreover, organized cell divisions were also found in the *EgTCTP* transformant (Figure 25E). To ensure that *EgTCTP* promote cell proliferation, the average cell density in gene transformed callus of oil palm was 4 fold higher than control and vector transformed calli which was 23.3, 27.7 and 98 cells in 1 mm^2 area, respectively (Figure 26).

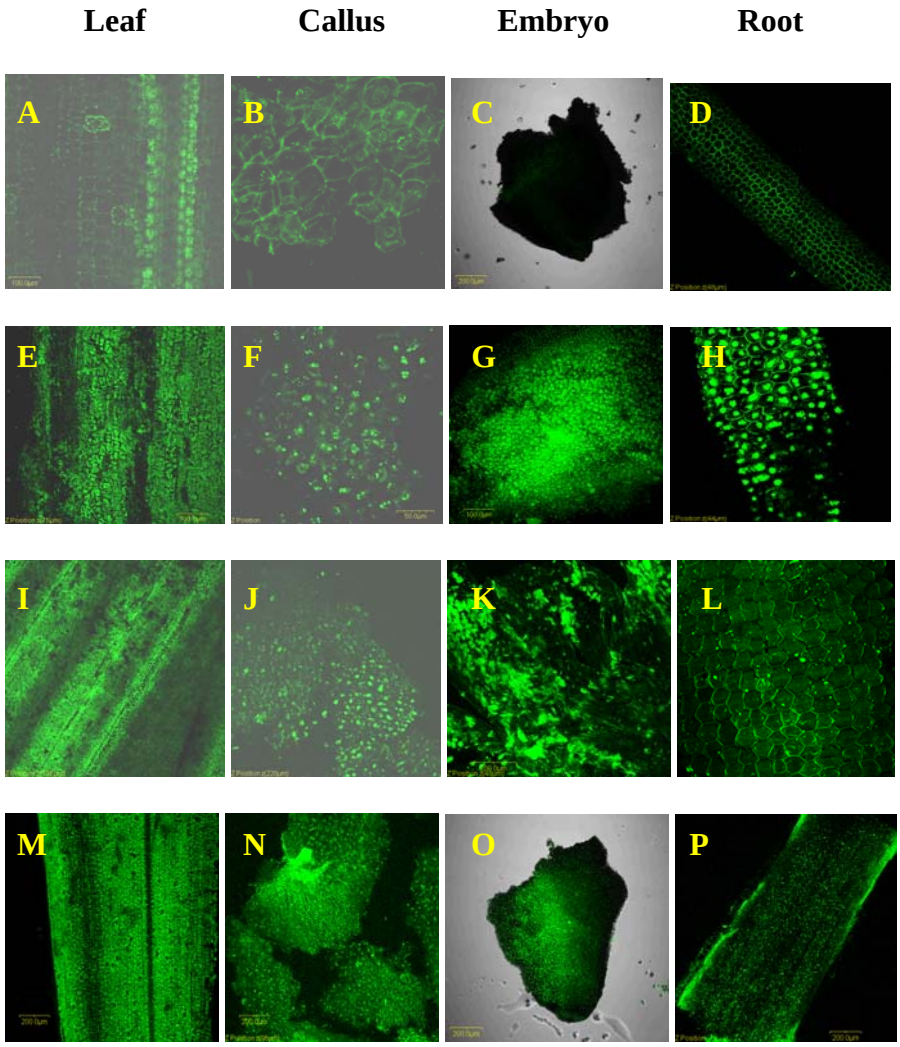


Figure 22 GFP expression in the transformed oil palm explants (leaf, callus, embryo and root) compared to the control (A-D), transformants 3 days after bombardment (E-H), transformants 1 month after bombardment (I-L) and transformants 6 months after bombardment (M-P) showed many bright green spots under a confocal laser scanning microscope.

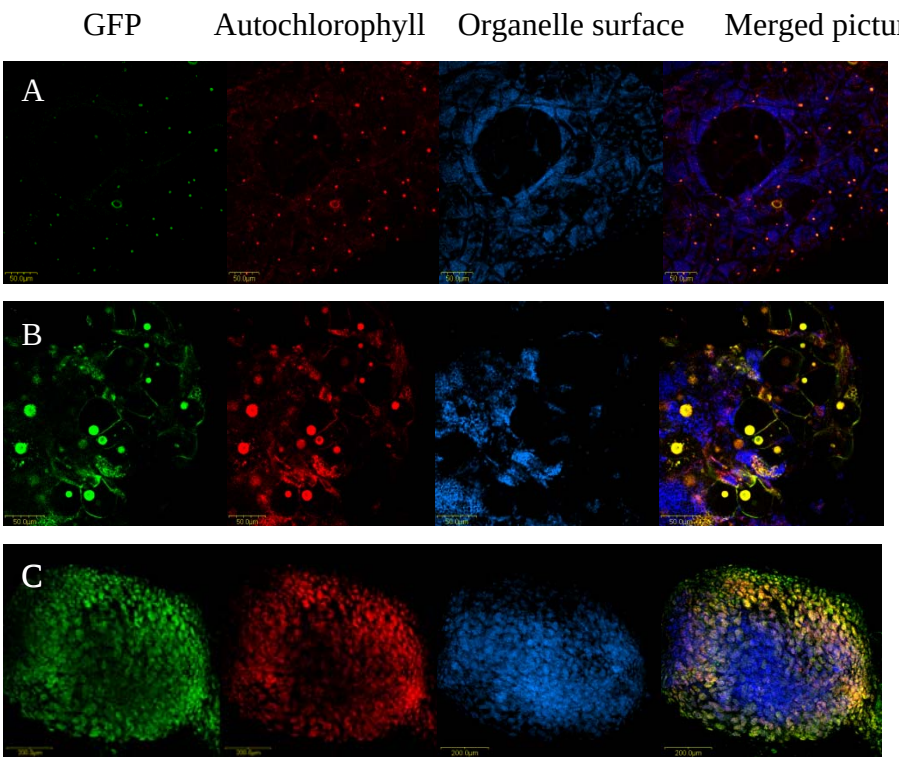


Figure 23 Confocal images that localize GFP in *Phalaenopsis* PLBs 12 days after bombardment. 35S CaMV-GFP expressed from pCAMBIA1302 vector. (A) control (B) vector and (C) gene.

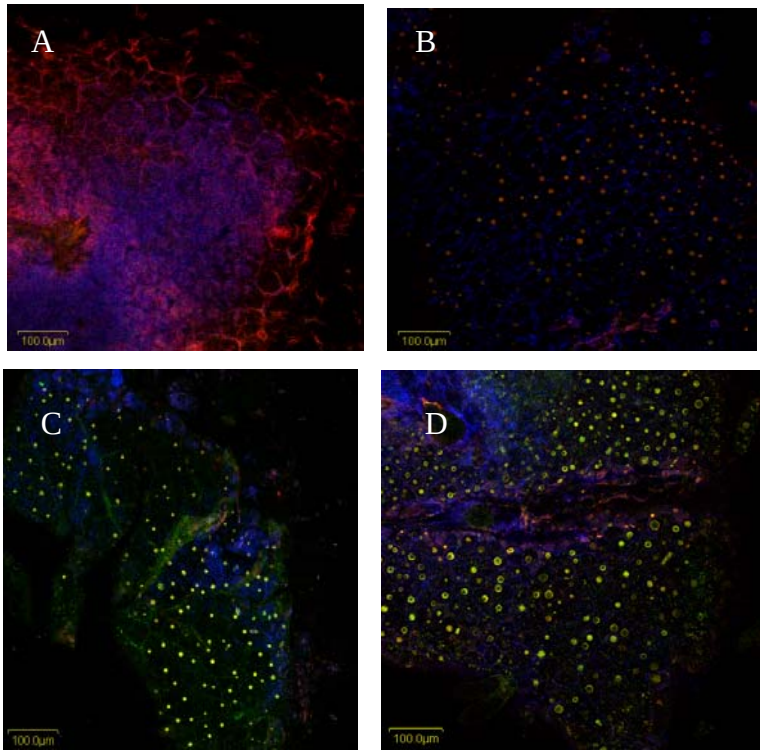


Figure 24 Confocal images that localize GFP in *Phalaenopsis* PLBs 60 days after bombardment. 35S CaMV-GFP expressed from pCAMBIA 1302 vector. (A) control : umbombarded (B) control: bombarded without vector and gene (C) bombarded with vector and (D) bombarded with transgene.

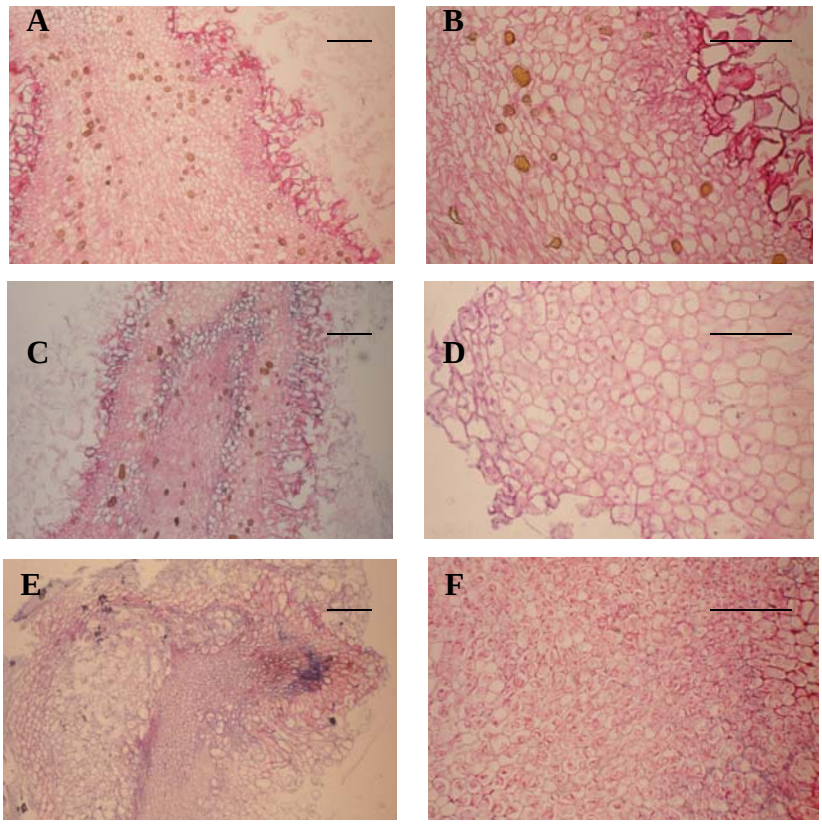


Figure 25 Longitudinal section through callus proliferation of oil palm 2 months after biolistic transformation. Control callus at x40 (A) and enlarged at x100 (B), Vector transformed callus at x40 (C) and enlarged at x100 (D), Gene transformed callus at x40 (E) and enlarged at x100 (F). (Bar = 100 µm.)

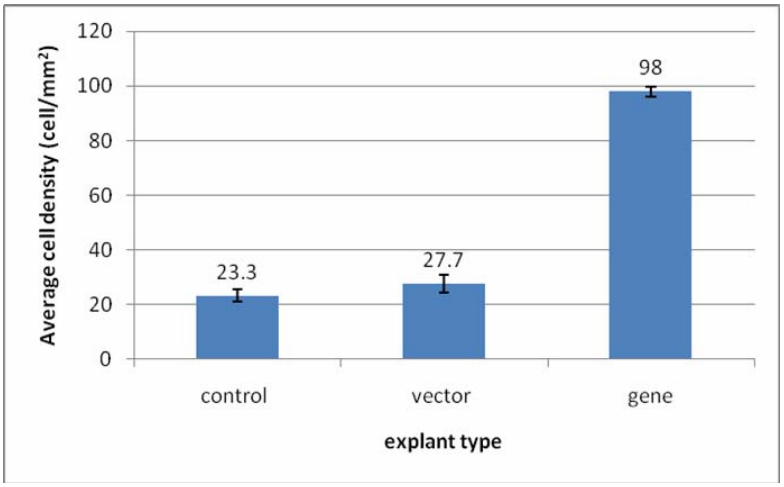


Figure 26 Cell density comparison in three types (control, vector, transformants and gene transformants) of oil palm callus.

3.7 PCR analysis of transformants

Two months post bombardment, all transformed explants were screened for detecting the presence of *mgfp5* and *EgTCTP* genes using PCR amplification of fragments in the transferred genes. The chromosomal DNA of oil palm and *Phalaenopsis* explants were isolated and used as the template in the PCR reactions later. Results of PCR reactions from oil palm and *Phalaenopsis* PLBs are shown in Figure 27 and 28, respectively.

In oil palm explants, an integrated fragment of *EgTCTP* was obtained at 600 bp (Figure 27, lane 4-6). This expected band was amplified in the *gfp* expressing callus, whereas no amplification was detected in the non-transformed (control) tissues (Figure 27, lane 2-3 and 7).

In transformed *Phalaenopsis* PLBs, the presence of *mgfp5* transgene produced the expected band at 362 bp whereas the fragment at 887 bp is specific to recombinant *gfp-EgTCTP* gene. The positive bands of *gfp* gene were seen in Figure 28 (lane 8-9) while the positive band of *gfp-EgTCTP* gene was detected in Figure 28 (lane 13). Nevertheless, no amplification was detected in the non-transformed orchids (Figure 28, lane 6-7, 10-12).

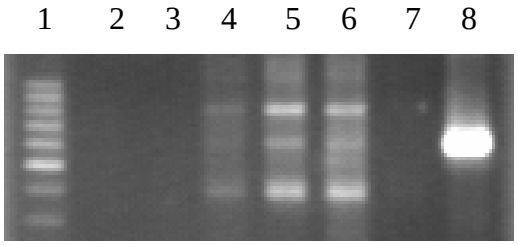


Figure 27 PCR analysis of transformed oil palm. Chromosomal DNA was amplified with the specific primer to *gfp-EgTCTP* gene. Lane (1) molecular marker DNA, lane (2) negative control, lane (3) vector alone transformed in seedling, lane (4) transformed seedling with *gfp-EgTCTP*, lane (5) transformed callus with vector alone, lane (6) transformed callus with *gfp-EgTCTP*, lane (7) negative control and lane (8) positive control.

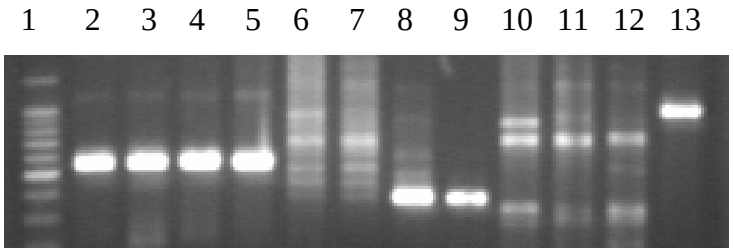


Figure 28 PCR analysis of transformed *Phalaenopsis* orchid. Chromosomal DNA was amplified with the specific primer to 18s rRNA (Lane 2-5), *gfp* (Lane 6-9) and *gfp-EgTCTP* (Lane 10-13) genes. Lane (1) molecular marker DNA, lane (6, 10) negative control (untreated orchid), lane (7, 11) negative control transformed with microcarrier only, lane (8, 12) transformed with vector alone, lane (9) positive control transformed with *gfp* gene and lane (13) positive control transformed with *gfp-EgTCTP* gene.

CHAPTER 4
DISCUSSION

4.1 Chitosan effect on oil palm regeneration experiment

It is well documented that vegetative propagation of palms proceeds from a callus phase to the production of plantlets and oil palm embryo is capable of dedifferentiating to form callus, which in turn, retains the potential to form plantlets. Callus production with generative capacity from excised zygotic embryos was observed in several palm species such as coconut palm (Fisher and Tsai, 1978; Gupta *et al.*, 1984), date palm (Gabr and Tisserat, 1985; LeThi *et al.*, 1999), Christmas palm (Srinivasan *et al.*, 1985), sago palm (Alang and Krishnapillary, 1986). In this study, sustained callus growth derived from embryos was attained on 2, 4-D containing medium. It is clear that 2, 4-D, the most commonly used auxin for embryoid induction, is required for callus initiation. In the previous study, embryogenic calli in oil palm embryos were induced on MS medium supplemented with 30 mg/l NAA or 3 mg/l 2, 4-D (Patcharapisutsin and Kanchanapoom, 1996). Numerous embryoids were produced after these embryogenic calli were subcultured several times to similar medium with an increase of NAA and 2, 4-D to 70 mg/l and 5 mg/l, respectively. For embryo development and plant conversion, these embryoids were transferred to medium devoid of 2, 4-D and supplemented with 15% (v/v) CW. In addition TDZ, a substituted phenyl urea, had a tendency to turn callus to non embryogenic one and this result is in agreement with Rajesh *et al*. (2003).

In this study, transfer of calli to chitosan supplemented medium stimulated the induction of organogenesis and subsequent plant regeneration has been presented. Chitosan is a major component of the shells of crustacean and the second most abundant natural biopolymer after cellulose. Chitosan is now widely produced commercially and has been documented to stimulate plant growth (No *et al.*, 2007). The effects of chitosan on the growth and development of *Dendrobium phalaenopsis* (Nge *et al*., 2006) and *Dendrobium* 'Eiskul' (Pornpeanpakdee *et al*., 2005) orchid protocorms in culture were reported. In the present study, the

exogenous application of chitosan induced shoot apex differentiation from the embryo derived callus suggesting that the role of chitosan might provide the stimulus effect. However, a longer time on chitosan containing medium led to the flower malformation and inhibited root development. Corley *et al*. (1986) showed that the incidence of mantled flower (the flowers are non-functional and there is no fruit development) in oil palm culture increases with the time cultures that have been maintained at the embryoid multiplication stage. Prolonged culture on media containing high levels of plant hormones may disrupt the normal hormone metabolism of the cells in some extents. In this study flower malformation is probably due to time in culture or chitosan application. Hence a better understanding in elucidating of the mechanism of chitosan and further studies on different sources of chitosan either derived from fungal or shrimp could improve the efficiency of organogenesis in oil palm.

Furthermore, *EgAP2-1* codes for an AINTEGUMENTA-like (AIL) protein was also enhanced regeneration capacity in transgenic *Arabidopsis* plants (Morcillo *et* al., 2007) therefore it might be involved in somatic and zygotic embryogenesis. In order to increase the regeneration capacity of oil palm directly, co-bombardment of *EgTCTP* transformants with the *EgAP2-1* gene could be performed.

4.2 Evaluation of helium pressure and type of oil palm explants using in biolistic gene transfer

In the present investigation, helium pressure and oil palm explant type using in biolistic transformation which studied in several reports (Parveez *et al*., 1997; 1998; Ramli and Abdullah, 2003; Abdullah *et al*., 2005) were tested to confirm the transformation efficiency. When cultured on the HYG selection medium, all of the controlled explants which unbombarded or bombarded with only gold particles died while only transformed tissue survived. This result confirmed the reliability of the medium selection. Tissue bombarded with the pCAMBIA vector and *EgTCTP* gene at all helium pressure levels produced a 55-100% survival of HYG resistant explants. These survival percentages of explants in the selective medium were used to monitor transformation efficiency. The lowest transformation percentage of 55

and 60%, for embryo and seedling, respectively were too low. However, a satisfactory 75.9% transformation efficiency was obtained for seedling explants at 1550 psi helium pressure. This result supports the previous experiments of Ramli and Abdullah (2003), who found the highest GFP expression in oil palm roots when bombarded at 1550 psi. In contrast, the highest transformation efficiency of 81.8% was obtained for embryos that were produced after bombarded at 850 psi. This was also in accordance with previous reports on transformation of oil palm utilizing immature embryos showed that the bombarded at 900 psi helium pressure was the best because it produced less damage and was amenable to both direct gene transfer (Lee *et al*., 2006) and *Agrobacterium*-mediated gene transfer (Abdullah *et al.,* 2005). By far the best transformation efficiency of 100% was obtained from callus explants after bombarded at 850 and 1550 psi, however, the high efficiency were also observed in 1100 and 1300 psi bombardment pressures. These results are in consistence with Lee *et al*. (2006) who reported that explants pre-treated with 2,4-D prior to transformation at every helium pressure induced rapid cell division could play major role in ensuring higher transformation efficiency.

From these data, it is possible to conclude that the mature embryo and young seedling is not as suitable for gene transformation as the callus. Callus bombarded at 850 and 1550 psi was suitable for efficient biolistic transformation. This condition with an oil palm callus should become the standard protocol for biolistic transformation of oil palm, as the 100% efficiency obtained here is a significant improvement on all previously attempted oil palm gene transformations.

To date, nanotechnology may influence in gene delivery and gene expression in plants because protamine which formed a nano-scale complex instead of the conventional spermidine coating could improve the particle bombardment efficiency in rice and maize suspension cells (Sivamani *et al.,* 2009). Furthermore, in the interesting work of Lee, *et al*. (2006), antibiotic selection was not used, even though their construct contained the *hpt*II gene. The additional stress inflicted by dying cells that might reduce the nutrient supply to transgenic cells or by producing toxic compounds that would further impede the proliferation of transgenic cells and their differentiation into transgenic plants, is the reason why an antibiotic selective medium was not used in the experiment (Ebinuma *et al.,* 2001).

4.3 Growth rate analysis of oil palm after biolistic transformation

The present report with 100% efficiency is a significant improvement for oil palm transformation. Furthermore in this experiment, a recombinant vector with *EgTCTP* gene was introduced into oil palm explants via the biolistic method. According to the invention of Kang *et al*. (2005), the *TCTP* gene could be a good tool for the genetic manipulation of plant growth rate. Therefore the main objective of growth rate measurement in explants was to evaluate the *TCTP* gene function as a regulatory role in cell proliferation (Bommer and Thiele, 2004). The expected transgenic explants with the *EgTCTP* gene should grow much faster than the controlled explants. As expected, both the *EgTCTP* transformed calli bombarded at 1100 psi and the 1 year and 6 months transformed seedlings bombarded at 1300 psi helium pressure were significantly different in growth rate among control, vector and gene groups. However, the growth rates of the gene group in callus explants were the highest value when compared to the control and vector groups at every bombarded helium pressure. In contrast, surprisingly, the transformed mature embryos of oil palm bombarded with the *EgTCTP* gene at every bombarded helium pressure grew slower than the control and vector groups. The results described here showed that mature embryo was not suitable to the biolistic transformation in oil palm species. For the supporting reason the transformation of mature embryo in oil palm should be continuing investigated. Nevertheless, in almost previous reports on biolistic transformation of many monocotyledonous crops (Aulinger *et al*, 2003; Huber *et al*, 2002) utilized immature embryo instead of mature embryo to achieve the successful result. This may be due to the plant regeneration ability after callus induction. Moreover, the presence of *EgTCTP* in transgenic seedling can accelerate the growth rate of mature seedlings than the young seedlings when compared to the non-transformed seedlings at 1300 psi helium pressure. This result supports the former experiment (Ramli and Abdullah, 2003) which found the highest GFP expression in oil palm leaf when bombarded at 1350 psi.

The conditions of helium pressure for biolistic transformation in oil palm callus were optimized. It revealed that all four helium pressures did not significant influence transformation efficiency. This was supported by high number

of callus growth at 1100 and 1300 psi. Although transformation efficiency was increased at 100% at 850 and 1550 psi but the growth of callus was less comparable to helium pressure at 1100 and 1300 psi, this could be due to the damage of tissues during the bombardment as in the case of 1550 psi. However, in the case of 850 psi, the callus growth was not influenced and this is in contrast to the findings of Abdullah *et al*., (2005) who reported that best result was obtained at 900 psi. One plausible explanation could be that the type of explants was different since optimum helium pressure was considered to be cell and tissue type dependent (Iida *et al*., 1990).

Generally, this observation clearly shows that the successful transformations for introducing the DNA into oil palm were callus which bombarded at 1100 psi helium pressure and young seedlings which bombarded at 1300 psi helium pressure. This condition should be the standard protocol for biolistic transformation in oil palm callus. On the other hand, the mature embryo was not suitable for biolistic gene transformation.

4.4 Regeneration evaluation of *Phalaenopsis* PLBs after bombardment

Biolistic transformation is normally used in orchid gene transformation because of its higher transformation efficiency than *Agrobacterium*-mediated transformation (Chai and Yuhao, 2007). PLBs of several orchid genera such as *Dendrobium* (Chia *et al.* 1994; Yu *et al*., 1999; Men *et al*., 2003), *Phalaenopsis* (Anzai *et al.,* 1996), *Cymbidium* (Yang *et al*., 1999) and *Oncidium* (Yee *et al*., 2008) were widely used as target tissue for particle bombardment to recover transgenic orchids. Moreover, *Agrobacterium*-mediated transformation method was also used orchid PLBs (Chai *et al.*, 2002; Liau *et al*., 2003; Chan *et al*., 2005) since the origin of orchid PLB is a single somatic cell, have high capability of regenerating into new plant, easy to root, presumed to be genetically uniform and can be induced efficiently from various somatic tissues including young leaves, stem segments (Sanjaya and Chan, 2007; Yee *et al*., 2008).

As far as the regeneration properties are concerned, the *EgTCTP* transformed *Phalaenopsis* PLBs using the optimal helium pressure at 1100 psi promoted the regeneration frequency and efficiency as well as shorten time of shoot

primordial initiation and produced more regenerated shoots per PLBs. In an evaluation of the number of regenerated shoots per explant, the results also showed that bombarded the explants with *EgTCTP* gene resulted in the highest shoot number compared to the control and vector group. For regeneration efficiency evaluation, the status of *EgTCTP* orchid transformants were in the above average level. These results were in agreement with the former oil palm result which *EgTCTP* transformed callus bombardment at 1100 psi gave the highest growth rate. Generally, from these investigations our results clearly demonstrated the importance of *EgTCTP* role which seems to use to assist with the transformation and provide benefits for plant growth and regeneration.

4.5 Visualization of GFP expression

GFP monitoring was an effective tool in screening explants at various stages of recovery because *GFP* gene offers the advantages to produce transgenic plants that do not contain antibiotic resistance genes for transformant selection and can be used successfully to avoid the destructive *GUS* assay for identification. The *gfp* expression was in agreement with Tee and Maziah (2005) who reported that the *gfp* expression rate transformed in *Dendrobium* Sonia 17 was higher than that for GUS under the control of the same promoter based on the GUS or GFP spot counts.

It was relatively easy to establish if transformation had occurred or not by looking for the green fluorescence because GFP was part of the pCAMBIA 1302 plasmid. Almost bombarded seedlings which were separated into leaf and root parts, calli and embryos of oil palm as well as PLBs of orchid showed many bright green spots from the GFP fluorescence inside the cells compared to the controls due to GFP expression under control of the strong constitutive CaMV 35S promoter can be observed in every plant organ where the 35S promoter is active (Harper and Stewart Jr, 2000; Parveez and Majid, 2008). Normally, the brightness of fluorescence was maintained at full intensity during the subculture. This indicated that explants and their plasmids were able to proliferate under HYG selective conditions. However, interference of chlorophyll with GFP fluorescence was a major complication for evaluation in plant research.

In oil palm leaf and orchid PLB, the red autofluorescence of the chlorophyll interacted with the green fluorescence of the GFP in order to make the plants transformed with *gfp* appeared yellow under UV light. By preventing autochlorophyll fluorescence in leaves of oil palm and orchid PLBs, the sequentially scanning of the triple color images method could be easily detected the difference between GFP fluorescence and autofluorescence of chlorophyll. Furthermore, there are other methods distinguishing GFP from endogenous autofluorescence such as using the optimized filter sets, dual-wavelength differential fluorescence correction, fluorescence polarization, use of image analysis software, autofluorescence quenching, the use of spectrally different GFP mutants, etc (Billington and Knight, 2001). Thus, the GFP-based visual selection has made it possible and simple to detect and select transgene-carrying tissues.

In general, wild type GFP was localized to the nucleus and cytoplasm of diverse plant cells (Haseloff *et al*., 1997; Arnim *et al*., 1998). Nevertheless exact localization of the *gfp* gene to the targeted organelle (mitochondria, plastid and endoplasmic reticulum) was also reported (Majid and Parveez, 2007; Omar and Grosser, 2008). To our knowledge, Parveez *et al*., (2000) was the first user for *gfp* gene in oil palm transformation and achieved stable transformation when using embryogenic calli bombarded with a non-targeted 35S-SGFP-TYG construct. This mutated synthetic *gfp* gene construct showed the highest number of GFP spots. On contrary to studies of Majid and Parveez (2007) and Parveez and Majid (2008), the sGFPS65T gene was the most active in oil palm. The same results were also observed for sugarcane which the spot number and fluorescence brightness of sGFP(S65T) was higher than mGFP5 (Elliot *et al*., 1999). So, it is postulated that the sGFP(S65T) variant and derivatives are suitable for using in monocots (Stewart, 2001). However, a few GFP spots from sGFP(S65T) have retained their expression for more than 5 months after bombardment and in contrast with constructs driven by other promoters as HBT, ubiquitin which the number of spots and their brightness declined drastically after 3 days of bombardment and totally faded out after 2 weeks (Majid and Parveez, 2007).

In this study, for the first time, *mgfp5* gene was used in transformation of oil palm explants and *Phalaenopsis* PLBs with high efficiency. The ability of the above tissues to express GFP activities more than 8 months post-bombardment further indicated that the transgene had been successfully and stably integrated into the genome of the putative transformants (Majid and Parveez, 2007). Nevertheless, the stable integration of the *EgTCTP* gene after integrating into the cells of oil palm and orchid plants had further been proved via PCR analysis.

4.6 Histological analysis

To answer the question that the increasing growth rate of oil palm transformants occurred by increasing cell division or cell size, histological study was performed to discriminate the difference of the untransformed and transformed explants. From permanent slides, cell density of oil palm callus was 4-fold greater for the *EgTCTP* transformant than for the control and the vector transformants. These results confirmed the proliferation role of *TCTP* gene in plant and were in accordance with Berkowitz, *et al.* (2008) who reported the characterization of TCTP from *Arabidopsis thaliana* via RNA interference of TCTP showed slowly vegetation growth and smaller cell size of leaf and lateral root. In comparison, study on overexpression of *Cucurbita maxima* TCTP in tobacco explants resulted in organogenesis and regulating cell proliferation (Hinojosa-Moya *et al.*, 2008). It means, function of TCTPs in animals and plants were similar which related to proliferation and differentiation because the TCTP sequence conserved in diverse organism.

4.7 PCR analysis of transformants

In the past, several research studies in oil palm (Parveez *et al*., 2004; Abdullah *et al*., 2005; Omidvar *et al*., 2008; Parveez *et al*., 2008) and orchids (Chia *et al*., 1994; Anzai *et al*., 1996; Yang *et al*., 1999; Yu *et al*., 1999; Men *et al*., 2003; Tee and Maziah, 2005; Suwanaketchanatit *et al*., 2007; Yee *et al*., 2008) had shown the stable integrated of transgenes via biolistic transformation although some former studies in oil palm (Ramli and Abdullah, 2003; Lee *et al*., 2006) and orchids (Janna *et al*., 2006) showed only transient expression of transgenes.

Eventually, presence and integration of the *mgfp5* transgene via particle bombardment method were found in other plants genome as petunia (Garabagi and Strommer, 2000), papaya (Zhu *et al*., 2004) and high oleic sunflower (Mohamed *et al*., 2006).

With regard to PCR analysis in oil palm and *Phalaenopsis* explants in this study, the obtained bands confirmed the presence of *mgfp5* and *EgTCTP* sequences in both monocots. Therefore this observation proves the stable integration of the *EgTCTP* gene into both explants.

CHAPTER 5
CONCLUSION

Oil palm (*Elaeis guineensis* Jacq.) is known as one of the most recalcitrant species for tissue culture and genetic transformation. The main objective of this research was to establish an efficient and reproducible transformation protocol for achieving the stable gene transformation via particle bombardment method. Moreover, this study was the first time to transform the exogenous *EgTCTP* gene extracted from *E. guineensis* leaves into oil palm and *Phalaenopsis* orchid explants to validate the potential function of the *EgTCTP* gene in monocots plant.

The first step was to establish an efficient and reliable tissue culture protocol to obtain the regenerated plantlets from oil palm callus by optimization of plant growth regulator concentrations. Eventually, TDZ and chitosan were used in the experiments.

For the next step in biolistic transformation technique, helium pressure levels and types of explants were optimized in order to determine the most efficient transformation system. Later, on the basis of the transformation, the survival explants which selected in 50 mg/l HYG were counted by estimating the transformation frequency.

Finally, for *EgTCTP* transgene which bombarded into the plant nucleus of oil palm and *Phalaenopsis* orchid, cell proliferation and regeneration roles were tested. Furthermore, the *gfp* reporter gene in pCAMBIA 1302 and *EgTCTP* transgene were monitored to check the expression, presence and success of integrated transgene in plant tissues by histological and PCR analysis.

The main important results of the present investigation are summarized as following:

1. To study effect of chitosan and TDZ whether improve the regeneration rate of oil palm callus or not, 15 and 20 mg/l chitosan either with or without 5 mg/l 2, 4-D enhanced the shoot regeneration of oil palm callus even though the regeneration rate was quite low.

2. Embryogenic callus was the most suitable target tissues for the biolistic transformation in oil palm when compared to mature embryo and young seedling.
3. The optimized helium pressure level for particle bombardment in oil palm callus was 1100 psi while in oil palm seedling explants was 1300 psi helium pressure.
4. In these above optimized conditions, the biolistic transformation of oil palm embryogenic callus and seedling with an *EgTCTP* gene were demonstrated with the significant difference growth rate between the control and experiment samples group whereas no significant difference growth rate in the transformed mature embryo of oil palm. Moreover, histological analysis was used to confirm the cell number of transformants which was 4-fold higher in cell density than the control groups.
5. This transformation strategy is efficient for the transformed callus production although the regeneration rate of oil palm callus was rather low. However, the regeneration capacity of *EgTCTP* transformed PLBs of *Phalaenopsis* orchid was studied to confirm the proliferation and regeneration role of *EgTCTP* gene in monocots. For this purpose, regeneration evaluation recorded as a mean percentage of explants with shoot induction, time of shoot primordial initiation in days, number of regenerated shoots per explant and scoring system were tested to evaluate the regeneration efficiency and the obtained results were shown that *EgTCTP* gene played an important role in these characteristics.
6. To monitor the expression, presence and integration of the transgenes in the putative transformants of both oil palm and orchid explants, GFP green fluorescence spots of *mgfp5* gene which was the part of pCAMBIA 1302 vector were detected by CLSM in all explants. For stable assay, the presence of transgenes in the hygromycin resistant explants was confirmed by PCR. It indicated that the transgene was integrated in the genome of transformants.

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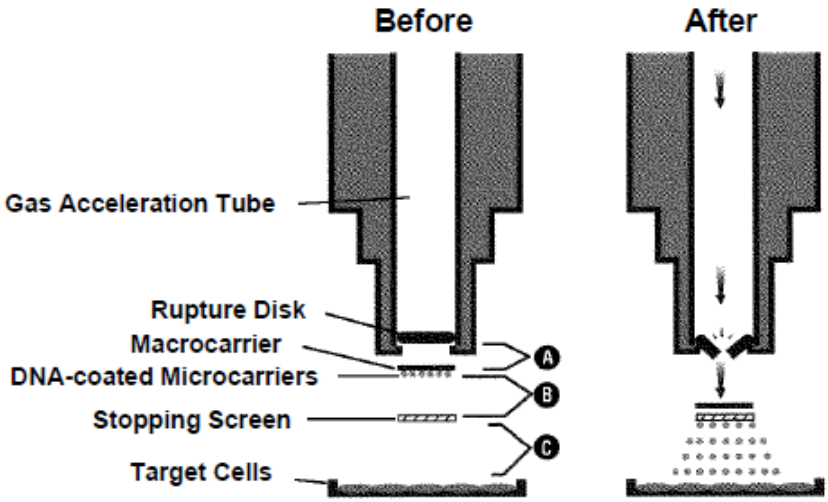
APPENDIX A

The biolistic bombardment process



The Biolistic PDS-1000/He system uses high pressure helium, released by a rupture disk, and partial vacuum to propel a macrocarrier sheet loaded with millions of microscopic microcarriers (tungsten or gold) toward target cells at high velocity. The microcarriers are coated with DNA for transformation. The macro-carrier is halted after a short distance by a stopping screen. The DNA-coated microcarriers continue traveling toward the target to penetrate and transform the cells.

The launch velocity of microcarriers is dependent upon the helium pressure (rupture disk selection), the amount of vacuum in the bombardment chamber, the distance from the rupture disk to the macrocarrier (A), the macrocarrier travel distance to the stopping screen (B), and the distance between the stopping screen and target cells (C).

APPENDIX B

Composition of Murashige and Skoog (MS) medium (1962).

Macroelements		
Ammonium nitrate, NH_4NO_3	1,650	mg
Potassium nitrate, KNO_3	1,900	mg
Calcium chloride, $CaCl_2 . 2H_2O$	440	mg
Potassium dihydrogen phosphate, KH_2PO_4	170	mg
Magnesium sulfate, $MgSO_4 . 7H_2O$	370	mg
Chelated iron		
Na_2-EDTA $. 2H_2O$	37.3	mg
Ferrous sulfate, $FeSO_4 . 7H_2O$	27.8	mg
Microelements		
Boric acid, H_3BO_3	6.2	mg
Manganese sulfate, $MnSO_4 . 4H_2O$	16.9	mg
Zinc sulfate, $ZnSO_4 . 7H_2O$	6.14	mg
Potassium iodine, KI	0.83	mg
Sodium molybdate, $Na_2MoO_4 . 2H_2O$	0.25	mg
Copper sulfate, $CuSO_4 . 5H_2O$	0.025	mg
Cobaltous chloride, $CoCl_2 . 6H_2O$	0.025	mg
Growth factor		
Myo-inositol	100	mg
Amino acid		
Glycine	2	mg

APPENDIX B (Continued)

Vitamins

Thiamine HCl	0.1 mg
Nicotinic acid	0.5 mg
Pyridoxin HCl	0.5 mg