CHAPTER I:

INTRODUCTION AND LITERATURE REVIEW

1.1 Oil palm origin and characteristics

1.1.1 Botanical characteristics of oil palm

The African oil palm, *Elaeis guineensis* is native to Africa, occurring between Angola and Gambia, while the American oil palm *Elaeis oleifera* is native to tropical Central America and South America. The African oil palm, *Elaeis guineensis* is placed in the Arecaceae family along with coconut and date palms. It has been elucidated the genetics of three naturally occurring fruit types includes *dura, tenera*, and *pisifera.* The *dura* (sh^+sh^+) is for the presence of a relatively thick shell (2-8 mm). The *tenera* (sh^+sh^-) with a relatively thin shell (0.5-4 mm). In comparison to *tenera* and *dura*, the *pisifera* (sh^- sh^-) is known for the absence of the shell (Hardon *et al.,* 1985). The popular cultivar is *tenera* which yield more oil concentration. Oil palm is a monocotyledonous plant that has the average height is about 20-30 feet in cultivation. The oil palm leaves are up to 25 feet in length containing leaflets around 200-300 per leaf. Leaf bases are persistent for years, and prominent leaf scars are arranged spirally on the trunk of mature palms where bases have fallen. A young tree produces about 30 leaves a year, the trees over ten years can produce 20 leaves per year. Oil palm is monoecious which enables cross-pollination because male and female inflorescence are produced from axillary buds separately to each other. The flowers are produced in dense clusters; each individual flower is small, with three sepals and three petals. Fruits are born on secondary branches, it has ovoid shape and elongate 2-5 cm in length, the weight varies between 5-20 g. Fruits are tightly attached to fruit bunches, the number of fruit per bunch is between 50 to 100 in young palms and up to 1000-3000 in older ones. Ripe bunches weigh from 20 to 50 kg (Hardon *et al.,* 1985). Oil palm takes roughly 7 years to bear fruits. Oil palm fruit takes six months to mature; when fruits ripen, the color turns from black to orange, the degree of darkness depending on light exposure and cultivar. Oil palm fruit includes four parts, the outer layer is the

pericarp that has a tough surface to protect the inner ones. The next layer is mesocarp that is rich in oil from which the industrial products are manufactured, used mainly for producing cooking oil. The endocarp or the shell is next, it usually appears as a black firm layer that covers the last part - kernel, the oil extracted from this part is normally for processing industrial foods. The annual harvested production for each hectare of oil palm is about 10 tonnes of fruits, which yields 3,000 kg of pericarp oil, and 750 kg of seed kernels, which yield 250 kg of extracted kernel oil as well as 500 kg of kernel meal. The meal is used for livestock breeding. Some varieties have even higher productivities which can be used for producing the vegetable oil used in biodiesel.

1.1.2 The use of oil palm

Oil palm has many applications in the industrial production, edible palm oil-based fats are primary element used to make processed food products such as cooking oil, shortening, margarines, ice creams and cookies. Palm oil specialty fats are also applied as a cocoa butter substitute in chocolates. Oil palm is now edible and has surpassed soybean oil as the most popular vegetable oil in the world with higher production (Table 1.1). Other products produced from oil palm are soap, washing powders and cosmetics, medicines and future diesel fuel substitute. These aforementioned products are produced from the crude palm oil derived from the mesocarp part. The kernel meal after extracting kernel oil is the secondary product is mainly used for animal feed. The relative high level of palmitate (about 44%) provide a stable level of sustainment that is suitable for high temperature frying application (Broun *et al*., 1999). The approximately 40% oleate and 10% linoleate of palm oil has been shown to lower total serum cholesterol, functioning in movement of cholesterol within the blood stream by reducing the level of the low-density lipoprotein cholesterol (Sundram *et al*., 2003). Additionally, the high level of the high-density lipoprotein and the removal of cholesterol from the bloodstream has been proven to protect against heart diseases (Sundram, 1997; Pedersen *et al*., 2005).

Table 1.1 Oil productivity of major oil crops.

Oil crop	Oil production (million tonnes)	% of total production	Average of yield (tonnes/ha/year)	Planted area (million ha)	% of total area
Soybean	33.58	31.69	0.36	92.10	42.24
Sunflower	9.66	9.12	0.42	22.90	10.50
Rapeseed	16.21	15.30	0.59	27.30	12.52
Oil palm	33.73	31.84	3.68	9.17	4.21
Total	105.94			218.02	

Source: Oil World

Palm oil's high nutritional qualities have been chosen as a superior ingredient in many types of foods. Research in United States has shown that palm oil is rich in both vitamin A and E. Recent studies also reveals that mesocarp oil rich in Vitamin A could be used as a powerful supplement in solving the Vitamin A deficiency problem that happens in children. Vitamin E antioxidants in palm oil are preferred choice in food and health products as an anti-aging and anti-cancer tool. Palm oil is also easily digested, absorbed and utilised in normal metabolic processes. Malaysia's liquid palm olein is a compulsory ingredient in cooking oil, which is sold under brands like Knife, Red Eagle, Labour and Carotino.

1.1.3 Oil palm migration from Nigeria to Malaysia and successful oil palm plantations in Malaysia.

The oil palm is a perennial crop that originated in the tropical rain forest of West Africa. It spread to South America in the 16th century and to Asia in the 19th century (Kajisa 1997). Oil Palm has been developed to be planted as a cultivar in Sumatra and Malaysia since early in the 20th century, cropped in Belgian Congo (now Zaire) around the 1920s and then other parts of West Africa. During the 1970s, Asia surpassed Africa as the crucial oil palm producing region in the world. Many other countries all over the world took part in producing palm-oil such as Nigeria, Indonesia, Zaire and the Ivory Coast. Among those, the tropical countries with a more suitable climate for oil palm development has been superior in the production of this type of fruit, for example Brazil, Colombia, Ecuador, Panama, Costa Rica, Papua New Guinea, Solomon Islands, New Britain, Thailand, Philippines. From

1970s, Malaysia planted and became the main producer of palm oil, in 1982 approximately 60% of the world production and over 80% of the world oil palm export (Table 1.2)

Table 1.2 World oil palm production from 1985 to 2005**.**

	1980	1985	1990	1995	2000	2005	2005
	Palm oil output x 1000 metric tonnes					**Share**	
Malaysia	2576	4133	6088	8123	10,842	14,962	44%
Indonesia	691	1243	2413	4220	7050	14,070	42%
Nigeria	433	386	580	660	740	800	2%
Thailand	–	–	232	354	525	685	2%
Colombia	–	–	226	388	524	661	2%
Papua New Guinea	–	–	145	223	336	310	1%
Cote D'Ivoire	–	–	270	285	278	260	1%
Brazil	12	29	66	75	108	160	0%
Others	875	1041	1000	5994	5191	1826	5%
World total	4587	6832	11,020	20,322	25,594	33,733	100%

Source: Oil World

Through the establishment of the Oil Palm Genetics Laboratory (OPGL) in the 1960's, Malaysia has expanded research on oil palm breeding and also carried out a large exchange program with Africa by the Malaysian Department of Agriculture. After that, the OPGL has been successful in increasing the yield of planting materials, thus making an impact in oil palm industry in the 1960s by commercialising its cultivation attractively. As the production is on, the government determined to control through legislation and implementation of programs by establishment of the Palm Oil Research Institute of Malaysia (PORIM) in 1979. PORIM's research was conducted in association with the private research stations owned by the large plantations. In order to develop industrial production and reach new technology frontiers, in recent years, PORIM has put a high priority on developing diesel and fat substitutes from palm oil as well as increasing the efficiency of planting materials though tissue culture.

Oil palm production in Malaysia today accounts for 3.7 million hectares of which over two million are in Peninsular Malaysia and the rest in the East Malaysian states of Sabah and Sarawak. Production is divided between many scales of production organization, large estates held by publicly listed companies, smaller estates are occupied by smallholders and government smallholder settler schemes. The industrial production in this country has undergone many obstacles and eventually now become the largest producer and exporter of palm oil over the world, accounting for 52 percent of world production and 64 percent of world exports in 1997.

1.2 Oil palm development and current problems.

1.2.1 History of Oil Palm Breeding Strategy

The first selection program of oil palm occurred in 1918 in Sumatra and continued to spread throughout Malaysia in thick-shelled *duras* (sh^+ sh^+). This material is originated from four palms in the botanical garden in Bogor (Java) planted in 1848. The figure 1.1 describes the distribution and multiplication of this material through Sumatra and Malaysia.
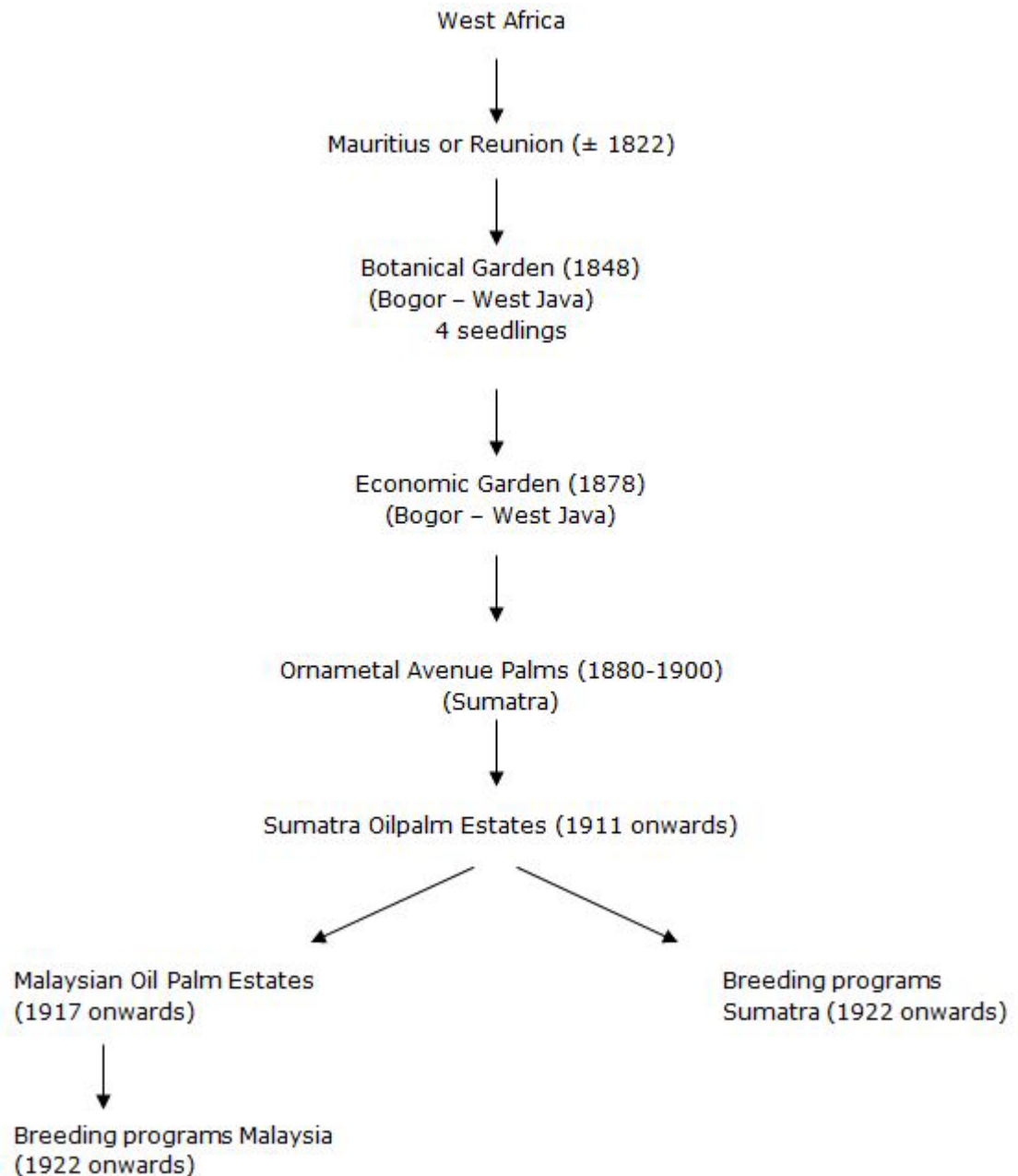
West Africa

↓

Mauritius or Reunion (± 1822)

↓

Botanical Garden (1848)
(Bogor – West Java)
4 seedlings

↓

Economic Garden (1878)
(Bogor – West Java)

↓

Ornametal Avenue Palms (1880-1900)
(Sumatra)

↓

Sumatra Oilpalm Estates (1911 onwards)

Malaysian Oil Palm Estates
(1917 onwards)

↓

Breeding programs Malaysia
(1922 onwards)

Breeding programs
Sumatra (1922 onwards)

Figure 1.1 The spread of 'Deli dura' from West Africa to Malaysia

The variety of *duras* has the relative amount of mesocarp per fruit that is determined under polygenic control and the original material that contains more mesocarp per fruit to most *dura* material was explored in the center of West Africa. Breeding programs started in Zaire, West Africa around 1922 and in Ivory Coast, Nigeria in the early 1930s. In Zaire the Institut National pour l'Étude Agronomique du Congo (INEAC), the breeding program started from open-pollinated seeds of one

tenera palm in the Eala Botanical Garden and nine *teneras* from Jewenda, N'Gazi and Isangi. By the process of mass selection, a small number of selected *teneras* that were superior than the others were chosen to be selfed and crossed. In their progenies, six *teneras* were selected and combined in a special crossing system. Inbreeding proved not to be effective in case of the predominance of the Eala palm in the parentage. The model *tenera* x *tenera* crosses could be reproduced by selecting *duras* in the selfed progeny of the one *tenera* with *pisiferas* in the selfing of the other *tenera* and vice versa.

The Institut de la Recherche pour les Huiles et Oléagineux (IRHO) in the Ivory Coast carried out the selected program as similarly to the INEAC after mass-selection programs in a limited *tenera* population had not been successful, then they turned into in-breeding (Gascon and Prévot, 1956). The program was conducted on both intra-origin crosses and crosses between origins by exchanging pollen. Crosses between Deli *duras* and African *teneras* yielded a better result than intra-origin crosses. The inter-origin which gained high yield in mesocarp oil amount was achieved when combining of a high bunch number in most of the African material, with high mean weight of bunches of the Deli *duras*. This breeding program of IRHO thrived in a form of recurrent reciprocal selection (Fig 1.2)

In Nigeria, the Nigerian Institute for Oil Palm Research (NIFOR) adopted the same approach to the IRHO program. The difference is the use of a relatively broader range of material from selected palms originated from various groves in Nigeria and some Deli *duras* from both Sumatra and Malaysia. The base material was not arranged following the origin, *duras* and *teneras* were classified into six groups based on being superior in limited sets of yielding and fruit characteristics. The aim is to produce seeds created by crosses between sets of parents with complementary characters. This strategy would seem to be suitable for traits that have the negative genetic correlations such as those were found between mean weight per bunch and the annual number of bunches per palm. Another interesting character is that this system may increase the genetic differences between population without necessarily inbreeding.

In Indonesia, the breeding strategy changed from *duras* to *teneras* was firstly based on *pisiferas* derived from experimental material in Zaire around the 1920s. Most of available material has been turned back to Eala palm is much of the INEAC (now INRA) material. The results from Zaire, Sumatra and Malaysia showed that the two

main characters determining yield, average bunch weight and number of bunches were inherited in an additive way in inter-population crosses. The conclusion is Deli *durasi* and INEAC *teneras* may constitutively adapt from selected populations that combine well, so the inter-origin superiority could be only a part of a story to explain for the unnecessary of further breeding. The recurrent reciprocal selection (RSS) strategy to oil palm breeding was questioned because only a few of the total number of palms can be tested adequately on their cross performance. Therefore it is implausible to recognize the maximum potential for specific combining ability between two populations whereas the objective of the test crosses between populations is to change into homozygosity for complementary genes. Palms which was selected to enter the test cross programs is based on their phenotypic performance and so they must possess the heterozygosity in the present material that related to yield and fitness. Consequently, RRS is not guaranteed to continue selection process, conversely the small number of plants that were used in the program may be the result of random loss of genetic variation. These results thrived Malaysian breeding program to widen genetic variation in base populations by inter-crossing materials originated from different sources. Family or individual palm plants that performed the general combining character were selected. Deli *duras* are considered as predominance despite the starting of some intercrossing with *teneras* from West African populations. The lines which exposed the superior in bunch weight or bunch number was selected. After *tenera* x *tenera* crosses, high–yielding *pisiferas* were taken out in progeny- tested in factorial designs, each *pisifera* crossed to a sample of *dura*.
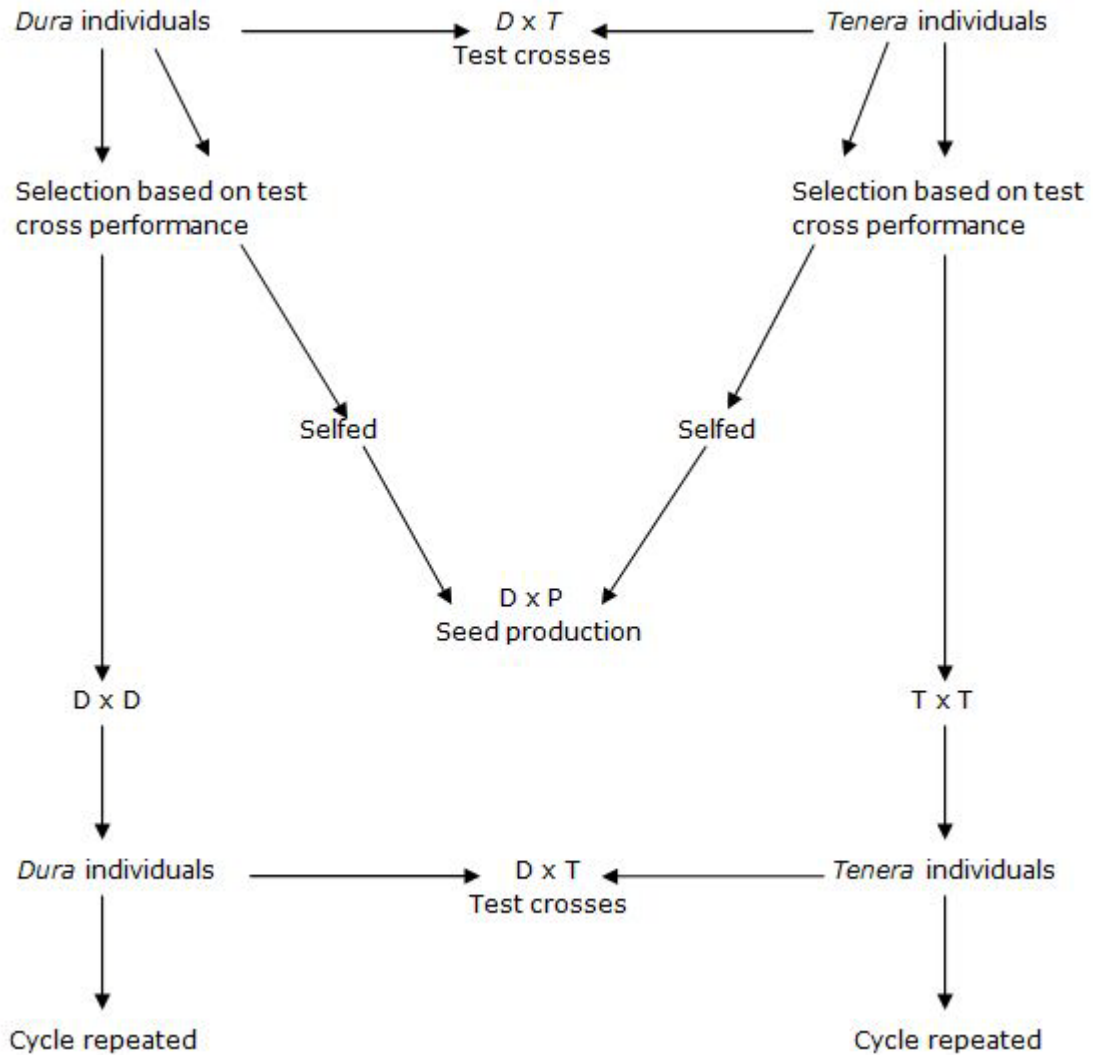
Figure 1.2 Reciprocal recurrent selection for oil-palm breeding

Hardon *et al.,* 1985 described the progeny-testing *pisiferas* on top of family selection yielded 7% selection advancement in terms of oil. To Deli *duras*, the phenotypic selection was not very effective which has the same result to the other studies describing that there is little additive variation for yields component left in this material. It was indicated that , on average, the performance of *dura* x *tenera* inter-origin test crosses could be reproduced in crossing *duras* in the selfing of one parent and *pisiferas* in the selfing of the other. In reality, current oil palm planting materials reproduced from those programs are still alike to each other because it is initially Deli *duras* crossed with *pisiferas* of exclusive (IRHO) or predominantly (Malaysia) West Africa origin.

Early selection has pointed out characters in family selection with low heritability such as mean weight per bunch and bunch number and individual palm selection for characters with relatively higher heritabilities such as mesocarp per fruit, percentage of shell per percentage of kernel. The objective is to increase the productivity of oil palm planting per hectare not to maximize oil yield per palm. Crop physiological studies indicated that selection for a high harvest index (HI) and tolerance to competition will achieve a higher planting density. Therefore, selection for HI was possible for family basis, and not effective for individual palm selection. Leaf area ratio (LAR) has a higher heritability than HI and that concurrent selection for HI and LAR will significantly increase HI. The following result of this selection is a reduction in vegetative vigour and height increment. Therefore, it is not suitable for breeding designed to exploit hybrid vigour. It is better for *dura* and *pisifera* selection should be carried out in single pooled populations which existing genetic variation to avoid inbreeding.

1.2.2 Efforts to improve oil palm characteristics.

Malaysian Palm Oil Board (MPOB), a biotechnology research centre in Malaysia, had the initial aim in genetic engineering for producing high oleate palms that is used for industrial feedstock and liquid oil market. The estimated value for high oleate palms is 1,500 USD per hectare per year if the oleic acid content is higher than 65%. The other recent targets in genetic engineering is to create high stearate palms as cocoa butter substitute (Sambanthamurthi *et al*., 2002).

The purpose of the program is to increase oleic acid and decrease palmitic acid in mesocarp oil. The amount of palmitic acid accumulated in oil palm is relatively high, 44% in total of the oil content. Based on the fatty acid composition of palm oil and the fatty acid biosynthesis pathway in oil palm, there are three significant points noted that β-ketoacyl-acyl carrier protein (ACP) synthase II (KAS II) activity is rate-limiting in the oil palm mesocarp, resulting in a 'bottleneck' of palmitic acid; thioesterase activity towards palmitoyl-ACP is very high, resulting in the release of palmitic acid and oil palm mesocarp contains an active stearoyl-ACP desaturase, thus most of the stearoyl-ACP formed is effectively desaturated to oleic acid (Parveez *et al.,* 2000).

High oleate and high stearate palms were produced through the genetic manipulation of the fatty acid biosynthetic pathways in the mesocarp as shown in

Figure 1.3. Biochemical studies, gene isolation and important enzymes were identified for the production of these fatty acids (Siti Nor Akmar *et al.*, 2001). A partial cDNA clone encoding acetyl-CoA carboxylase (ACCase), an enzyme catalyzes the first step in lipid biosynthesis, the flux-controlling enzyme has been isolated. The key enzymes for this genetic pathway in producing of high oleate and stearate palms are β-ketoacyl ACP synthase II (KAS II) and acyl-ACP thioesterase. KAS II is limiting in oil palm mesocarp, the method of improving is to increase the level of KAS II gene expression, it catalyzes the elongation from C16:0 to C18:0 and to antisense palmitoyl-ACP thioesterase for reducing the production of palmitate to increase oleate. Subsequent studies have revealed the function of necessary enzymes and full-length of cDNA have been sequenced and used in construct the mesocarp-specific promoter for the transformation process. The full-length cDNA clone for stearoyl-ACP desaturase has been isolated and the antisense form introduced into palms in order to increase stearic acid have been introduced.
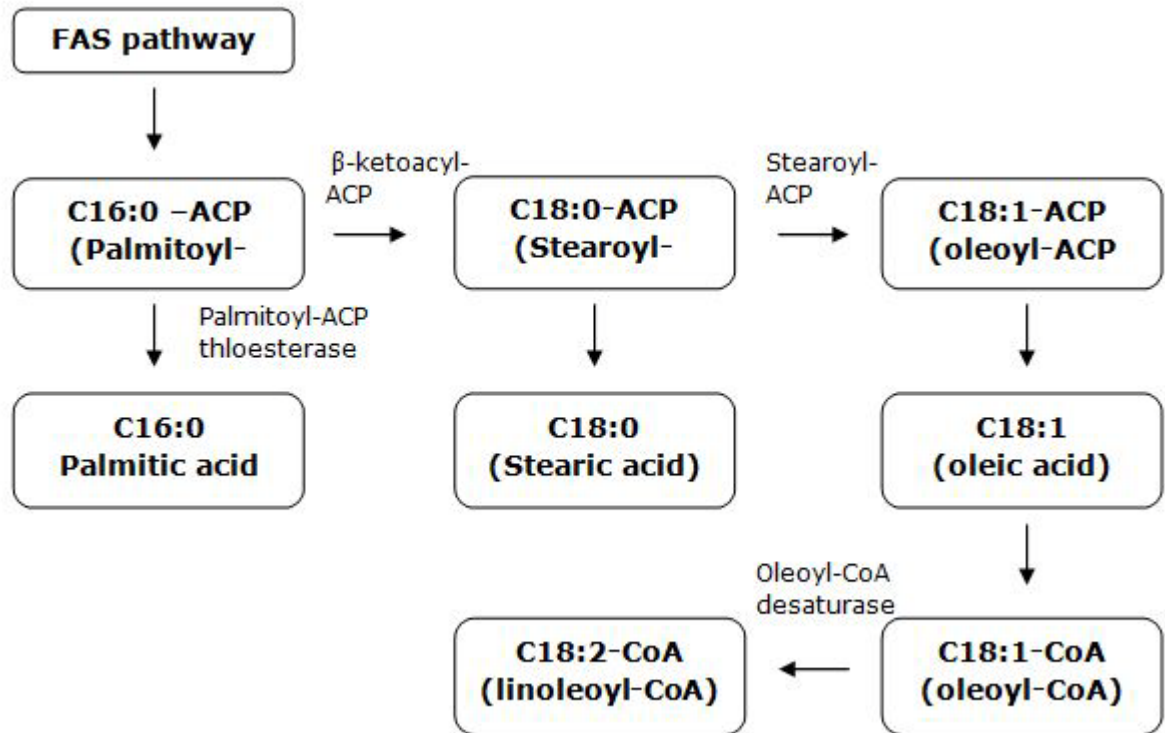

Figure 1.3 Fatty acid biosynthesis in plants (FAS= fatty acid synthase) (Modified from Basri 2004).

1.3 Overview of directly genetic transformation methods using in plant transformation.

A process of creating transgenic plant usually undergoes a complex multiple stage which follows this procedure. First, novel DNA is introduced by delivery into the host cell, this exogenous DNA is then stably integrated into the host plant genomes and express the novel traits and a viable transgenic plant is obtained. There are many transformation methods that were discovered and developed in recent decades, they were classified into two types of genetic transformations: direct gene transfer and indirect gene transfer. The direct gene transfer methods include: microprojectile bombardment (also known as biolistic and particle bombardment), electroporation, protoplast transformation and silicon carbide fibers. Meanwhile, the indirect gene transfer method employs usage of soil-borne bacterium *Agrobacterium tumefaciens*.

1.3.1 Biolistic or Particle Bombardment

Microprojectile bombardment or biolistic is a popular transformation method that was first introduced in 1987 (Primrose *et al*., 2001). In this method, firstly, a gold or a tungsten spherical particles that has approximately 0.4 to 1.2 micrometers (μm) in diameter are coated with DNA. This exogenous DNA has been previously precipitated with $CaCl_2$, spermidine, or polyethylene glycol. After that, the coated particles will be bombarded with high speed by helium pressure, its velocity is about 300 to 600 m/s. At first, it was bombarded by the gene gun , later particle bombardment apparatus (PDS 1000/He) was widely applied. The particle could penetrate into the plant cells without causing significantly damages to the cells with different adjusted distances that the particle has to go through before touching the plant cells. After bombarding into the plant cells, the DNA is removed from the particle and integrate into plant DNA. This method can be used to transfer foreign DNA into the type of explants. For example, callus cultures, meristematic tissues, immature embryos, protocorms, coleoptiles, and pollen in varieties of plants, including monocots and conifers.

The first experiment that used this parameter is intact onion epidermis was bombarded with tungsten particles coated in tobacco mosaic virus (TMV) RNA. After three days, the onion cells that contained 40% DNA from the particle proven the evidence of TMV replication (Sanford *et al.,* 1987). The number of transgenic

species produced by this transformation method was then greatly increased over the last ten years. Some notably successful crop plants that have been commercialized included rice (Christou *et al*., 1991), wheat (Vasil *et al.,* 1992), oat (Somers *et al.,* 1992), sugar cane and barley (Wan & Lemaux 1994).

In plant research, biolistic is a major method that is applied in transient gene expression studies, production of transgenic plants and inoculation of plants with viral pathogens. This method is a good choice for the study of transient expression 24-28 hours after bombardment as well as for plastid transformation (Bruce *et al.,* 1989). However, biolistic has some disadvantages. The transformation efficiency is lower than *Agrobacterium*-mediated transformation and the experimental facilities of biolistics are more expensive than other methods. Transgenic plants obtained by biolistic transformation usually expose a complex pattern of transgene insertion that could cause gene silencing and variation of transgene expression. Long fragment of DNA is difficult to be introduced because truncation may occur in the integration process.

1.3.2 Electroporation.

Electroporation is a rapid and simple technique to deliver DNA into plant cells and protoplasts. The materials are incubated in a buffer solution involving DNA and exposed to high-voltage electrical pulses. The DNA then migrates from the high-voltage-induced pores in the plasma membrane and integrates into the genome. It has been found that when subjected to the electric pulses the cells may take up exogenous DNA from the suspending solution. The successful transformed cells can be selected based on the selectable marker after stable transformation. Some crop species that have been successfully transformed using electroporation are all the major cereals such as rice, wheat and maize. In this technique, protoplasts and varieties of intact cells and tissues for example, callus culture, immature embryos and inflorescence materials can be used. However, the pre- and post- plant materials were applied for this method must be treated specifically with buffer or high osmotic pressure.

The advantages of this method are specialized vectors are not required, expression items can be employed in standard *E.coli* vectors. It is useful for high efficiency transient expression of foreign genes in plants without the need of selectable marker. However, limitations are that it can only be used with protoplasts. The

frequency of stable transformation is low (0.001 or even less). In stable transformation, the integrated DNA is extensively rearranged with high copy number.

There are many factors that affects the efficiency of electroporation including temperature, electric field parameters (voltage, resistance and capacitance), DNA sample, various host-cell factors (genetic studies, growth condition, and post-pulse treatment). Subsequent studies have revealed that linear rather than the supercoiled, plasmid DNA and the presence of spermidine in the incubation buffer enhances the electroporation efficiency. The field strength (the voltage applied across the electrodes) is optimized from 0.5-2.0 Mhz at 100-400 V cm^{-1} of protoplasts contributes important part in the success of electroporation either. In case the amount of DNA delivery is low, resulting in low copy number of transgene in the transformants, the delivery rate (the number of electroporated cells receives DNA) is still high. However, the use of electroporation on pollen may cause the escape regeneration phase. Although it has some apparent advantages, it is not preferred as other transgenic method because the irreproducible transformed cells and non-Mendelian segregation of progeny.

1.3.3 Protoplasts transformation

Plant cells that have their cell walls removed are called protoplasts. The protoplasts can be transformed with naked DNA by using a chemical substance polyethylene glycol (PEG) in the presence of divalent cations (for example, calcium). The plasma membrane of plant protoplasts is destabilized by the chemical substance (PEG) and divalent cations added to the nutrient solution containing the protoplasts, so it is easy for the naked DNA to fuse with the protoplasts under the favored condition. Once invading into the protoplasts, DNA migrates to the nucleus and integrates into the genome.

The first two successful experiments using protoplast as explants were conducted on tobacco and petunia (Primrose *et al*., 2001). Protoplasts of a mutant, white colored petunia strain were transformed with recombinant plasmid that contained *nptII* gene, maize complementary DNA (cDNA) encoding the enzyme dihydroquercetin 4-reductase, which is involved in anthocyanin pigment biosynthesis by electroporation. After a few days, viable protoplasts from the kanamycin supplemented medium gave rise to microcalli, which could be regenerated into the

whole plants. The mutant of petunia flower produced that had the brick-red color instead of white gave evidence that the maize cDNA had been successful installed into the genome. After successful experiments in dicots, protoplasts was then established in monocots transformation. In the first trials of experiments on monocots, involving wheat (Lorz *et al.,* 1985) and the Italian ryegrass *Lolium multiflorum* (Potrykus *et al.,* 1985), transgenic callus obtained at first, but failed to regenerate transgenic plants. Most of monocots cannot regenerate from protoplasts shows that they lost their competence in response to tissue culture conditions as the cells differentiate. Therefore, regenerating a whole plant from protoplast is not easy for some species, it is the reason why protoplasts are rarely employed due to the low frequency of transformants. Additionally, DNA used for transformation has tendency to be degraded and rearranged.

1.3.4 Silicon carbide mediated transformation.

Silicon carbide is a recently developed method which is suitable to deliver DNA to plants that requires no specialized equipment. Physical and chemical features of silicon carbide fibers allow them to penetrate into the cell without killing them. Plant material such as cell in suspension culture, embryos or embryo-derived calluses can be introduced into a buffer storing DNA and silicon carbide fibers which is then vortexed. The fibers' diameter and length are 0.3 – 0.6 μm and 10 – 100 μm respectively, with the small size like that, the fibers are able to penetrate the cell wall and plasma membrane that allows DNA to invade to the inside of the cell. The demonstration of this technique is rapid, easy to set-up, cheap and effective on variety of cell types. Some of the disadvantages were recorded such as low transformation efficiency, cell damage and health concern on potential risk of inhalation of the fibers (Komatsu *et al*., 2006). The efficiency of this method depends on some elements such as the fiber size, parameters of vortexing, shape of the vessels used, plant species and explant, characteristics of the plant cells and especially the thickness of the cell wall (Racoczy-Trijanowska, 2002). Successful examples of silicon carbide mediated transformation were maize (Kaeppler *et al*., 1992), rice (Komatsu *et al*., 2006), wheat (Briside *et al*., 2000), tobacco (Kaeppler *et al*., 1990). Recently, the transformation of rice callus based on whisker fibers was to 30-50% (Komatsu *et al*., 2006). Silicon carbide is shown to be effective as compared to biolistics, so it may be chosen as an alternative option for soybean embryogenic tissue transformation (Terakawa *et al*., 2005). Silicon carbide fibers

are also used in corporation with *Agrobacterium* by causing wounds to improve the transformation frequency.

1.4 Agrobacterium tumefaciens – a useful tool of indirect gene transfer.

Agrobacterium tumefaciens belongs to the class of ɑ-proteobacteria, family Rhizobiaceae and is classified within the *Agrobacterium* genus. It is a soil-borne bacterium that causes crown gall disease. *A.tumefaciens* infects more than 90 families of dicotyledonous plants. The gall is the result of transfer, integration and expression of a special set of genes called T-DNA originated from the tumor-inducing (Ti) plasmid. When these genes are expressed, the plants synthesize plant growth hormones and bacterial nutrient source called opines. The transfer process of T-DNA is regulated by a set of virulence genes (*vir)* located within the Ti-plasmid. The T-DNA is called the transfer region that makes *A.tumefaciens* an effective tool for gene transfer in research of transgenic plants and production of genetically modified crops.

1.4.1 Ti plasmid

Ti-plasmids are about 200-800 kbp. It is classified depending on the opines (nopaline and octopine), which is the result of tumors they induce. Another bacterium called *Agrobacterium rhizogenes* which also belongs to the family Rhizobiaceae cause hairy root disease in plant. *Agrobacterium rhizogenes* possesses root-inducing plasmid called Ri plasmid. The Ri-T-DNA includes genes homologous to the *iaaM* (tryptophan 2-mono-oxygenase) and *iaaH* (indoleacetamide hydrolase) genes of *A.tumefaciens.* Ri T-DNA has four other genes are named *rol* for root locus. Two of these, *rolB* and *rolC*, encode P-glucosidases to hydrolyse indole- and cytokine-*N*-glucosides. When plants are infected by *A.rhizogenes*, root cells are abundantly proliferated at the infected sites. Hairy roots are important in plant biotechnology aspect because it can be cultured in vitro. For years, the *A.rhizogenes* have been used as a source of secondary metabolites from the production of pharmaceutical proteins. *A.rhizogenes* was considered as alternative strategy to *A.tumefaciens* for gene transfer as the production of hair roots could be regenerated into whole plants. This strategy seems to have been eliminated from the more efficiently that *A.tumefaciens* systems demonstrated.

1.4.2 T-DNA region

The T-DNA region is the transfer region that incorporates the DNA genetic elements between two borders into the plant genome. Ti – plasmid is defined by the presence of the left and right border, it includes nopaline strains or octopine strains. The nopaline strain contains one T-DNA about 20 kb, octopine strain contains two T-DNA regions called T_L (14 kb) and T_R (7 kb) (Fig 1.4). In octopine strains, T_L region carries the oncogenic genes, T_R functions in opine biosynthesis. Octopine Ti-plasmid has 8 open reading frames (ORFs) and possesses an overdrive sequence that maximize the T-DNA transfer capacity. Nopaline Ti plasmid has 13 ORFs similarly to eukaryotes.   The nopaline T-DNA and the T_L octopine T-DNA share the same characteristic in the core region which is known as a region contains genes that encode for functional proteins. These proteins regulate the hormone synthesis (auxin or cytokinin), opine synthesis and genes that determine the tumor size.
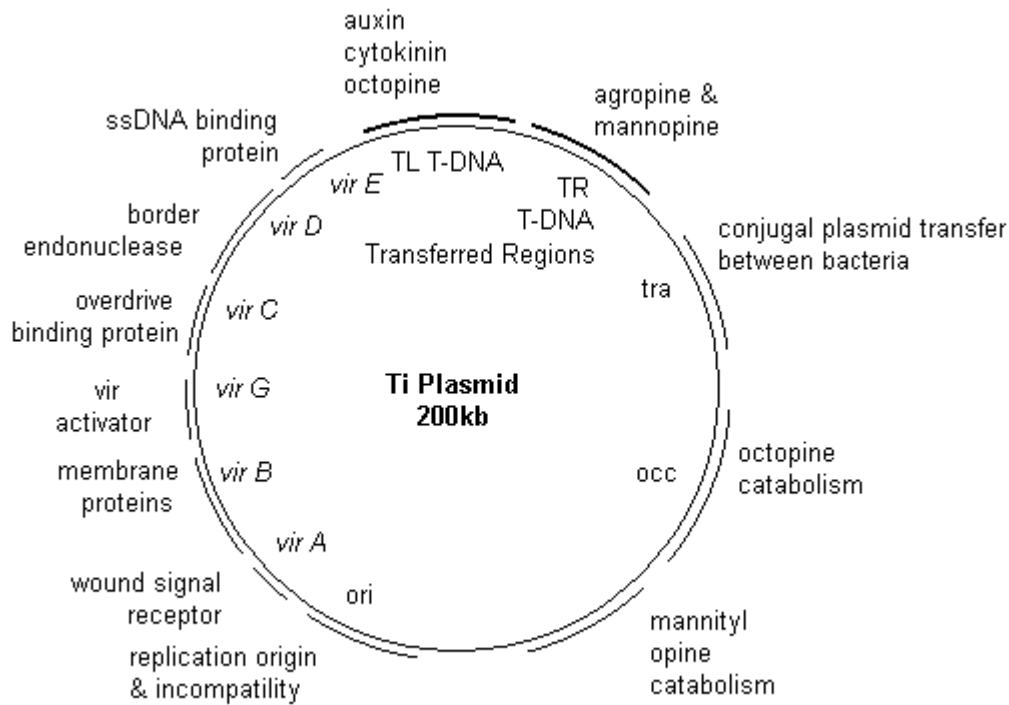

Figure 1.4 Genetic map of an octopine type Ti plasmid.

1.4.3 Vir region

Vir genes are arranged in the region of 35kb of Ti plasmid, outside the region of DNA. The vir gene's products are necessary for the transformation and the expression of the T-DNA into the genome of a plant cell. The vir region includes six genes: vir A, B, C, D, E and G (Table 1.3). VirA and virG are the only two monocistronic loci, the other four virs are polycistronic encoding several proteins. These genes regulate partially and function as a cascade of transcriptional events. The cascade begins with virA which is constitutively expressed. VirG transcripts the two mRNA molecules that differs at the 5' end of each other, one is constituently expressed and a longer which only is expressed when plant induces its chemical compounds. VirB and virE polypeptides are the two vir products that are induced in a relatively large amount by the wound that stimulates the plant in secretion the phenolic compounds.

Table 1.3 *Agrobacterium tumefaciens* chromosomal virulence and associated functions loci.

Vir Gene	Function
VirA	Play a role in encoding a protein which assembles transmembrane receptor found in bacteria.
VirG	A type of protein which transfers environmental information to other vir loci; requires *virA*; high effect on *virB , C, D, E*.
VirE	A single-stranded DNA binding protein that coat the T-strand during process.
VirC and *virD*	Endonuclease cleaves at the specific site at 25bp directly on the repeated borders of the T-DNA; produce a T-strand functions in transporting the genes into the plant cell.
VirB	Contributing a part in T-DNA transfer directly at the bacteria cell surface.

1.4.4 T-DNA Translocation Process

During the infection process, T-DNA is flanked by left and right border. Each border has 25 bp. The T-DNA possesses two types of genes: the oncogenes encoding for enzymes that synthesize auxins and cytokinins and is the cause of tumor formation; the other genes encoding for the synthesis of opines. Substances which are

synthesized by the opines are compounds of sugars and amino acids that are produced by the crown gall cells and absorbed by *A. tumefaciens* as carbon and nitrogen sources. The Ti-plasmid also contains the opine catabolism region that are induced by the crown gall cells, set of vir genes which are responsible for transfer from the bacterium to the plant cell and regions for bacterium-bacterium plasmid conjugative transfer (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995). The 30 kb virulence (*vir*) region is organized in six operons that are essential for the T-DNA transfer (*virA, virB, virD,* and *virG*) or for the increasing of transfer efficiency (*virC* and *virE*) (Hooykaas and Schilperoort, 1992; Zupan and Zambryski, 1995). Many chromosomal genetic elements function as an important role in the attachment of *A. tumefaciens* to the plant cell and bacterial colonization: the loci *chvA* and *chvB*, is responsible for the production and excretion of the β-1,2 glucan (Cangelosi *et al*., 1989); the *chvE* mainly function in sugar enhancement of the *vir* genes introduction and bacterial chemotaxis (Ankenbauer *et al*., 1990, Cangelosi *et al*., 1990, 1991); the *cel* locus, is known as the source of cellulose fibrils (Matthysse 1983); the *pscA* (*exoC*) locus, functioning in the synthesis of both cyclic glucan and acid succinoglycan (Cangelosi *et at*., 1987, 1991); finally, the *att* locus is responsible for the cell surface proteins (Matthysse, 1987).

The process of gene transfer from *Agrobacterium tumefaciens* to plant cells is briefly illustrated in figure 1.5, the process is described by main five stages: bacterial colonization, induction of virulence genes, initiation of T-DNA transfer, T-DNA transfer process, and integration of T-DNA into plant genome.
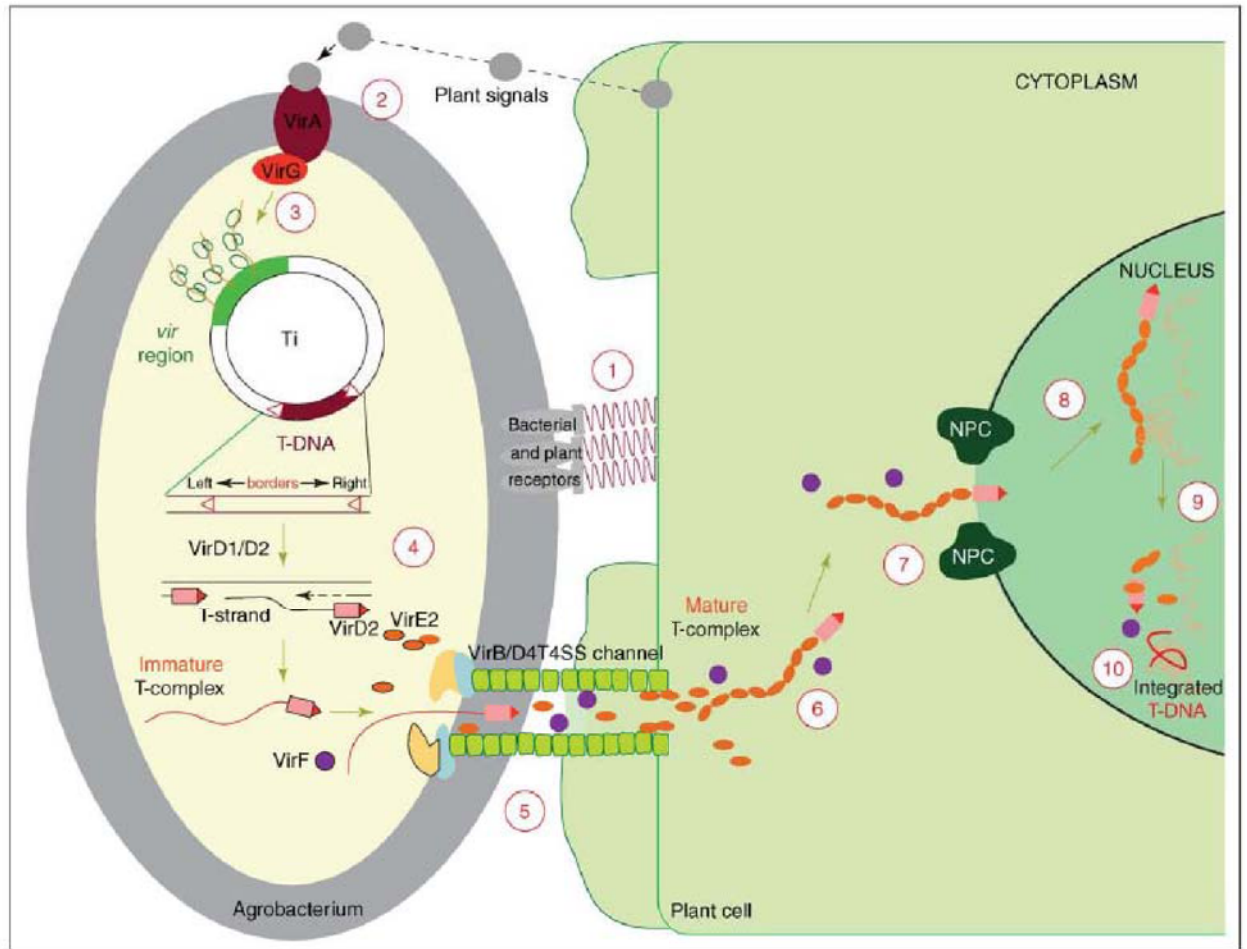
Figure 1.5 Hypothetical model on transformation process of *Agrobacterium tumefaciens* to plant cells (Tzfira *et al.*, 2006). The transformation process includes 10 steps and starts by the recognition and attachment of the *Agrobacterium* to the host surfaces (1) and the sensing of the signal by the *Agrobacterium* VirA/VirG two component signal transduction system (2). After that, the vir gene region is activated (3), the VirD1/VirD2 protein complex generates a mobile copy of the T-DNA (4) and released as an immature T-DNA protein complex (VirD2-DNA) with several other proteins into the host cell cytoplasm (5). After being associating with VirE2, the T-complex is now mature and migrates from the bacteria to the host cell cytoplasm (6) and is actively imported into the host-cell nucleus (7). Inside the nucleus, the T-DNA is now exposed to the point of integration (8), leave its transporting proteins behind (9) and integrated into the host genome (10) (Tzfira *et al.*, 2006).

Stage 1: Bacteria colonization

The bacterial colonization process is triggered by the wounds on the plant cell's surface. The chromosomal 20 kb *att* locus are responsible for attachment of the T-DNA to the plant cell. The extensive study on this locus using transposon insertion mutants show that insertion occurs in the left 10 kb side of this region will create an avirulent mutants that is able to recover its attachment ability when the incubation of wild-type virulent bacterium was included in the culture medium with plant cells. This makes the medium absent in the mutant strain that allows the attachment to the plant cell. Conversely, mutational insertion in the 10 kb right side of the *att* locus led to the penalty loss of attachment ability which is no longer recovered by conditioned medium (Bradley, 1997). These results suggest that genes at the *att* left side are responsible for molecular signaling events, while the right side are involved in the production of fundamental components. The *att* left side comprises nine open reading frames (ORF). Four of these ORF share similar characteristics to the genes involved in the periplasmic binding protein dependent (or ABC) transport system (Ames *et al*., 1990; Higgings *et al*., 1990).

Stage 2: Induction of virulence genes.

The 30-40 kb *vir* region encodes the genes that regulate the T-DNA transfer. This region includes at least six essential operons (*vir A, vir B, vir C, vir D, vir E, virG*) and two non-essential operons (*virF, virH*). Each operon has its own number of gene, for example, *virA, virG* and *virF* have only one gene; *virE, virC, virH* have two genes while *virD* and *virB* have four and eleven genes respectively. The two operons *virA* and *virG*, encoding for a complex (VirA-VirG) system that activates the transcription process of the other *vir* genes (Nixon, 1986; Iuchi, 1993). VirA and virG contain a two-component system that senses the phenolic compounds produced by the wounded plant cells. Wounding is particularly important in plant transformation, *Agrobacterium* has the capacity to sense a potential host that carries the wounds. The wounds contain the phenolic compounds that trigger the bacteria activity. VirA is activated by these phenolic inducers resulting in phosphorylation and activation of the VirG protein. The signals for VirA activation contains acidic pH, phenolic compounds, such as acetosyringone and some monosaccharides. VirA protein composes three domains: the periplasmic domain and two transmembrane domains (TM1 and TM2). The TM1 and TM2 domains act as a transmitter (signaling) and receiver (sensor) (Parkinson, 1993). The periplasmic

domain is necessary for monosaccharide detection (Chang and Winans, 1992). Staying adjacent close to the TM2 domain within the periplasmic domain are two regions, one strong hydrophilic and another hydrophobic regions (Heath *et al*., 1995). The TM2 is defined as kinase domain and responsible for activation of VirA (Huang *et al*., 1990), as a response to signaling molecules from the wounded regions.

Activated VirG connects to the vir gene operon that leads to the increased expression of each of these operons. Besides the induction of vir genes by phenolics, monosaccharides are detected by protein ChvE, encoded by a gene on the *Agrobacterium* chromosome act as co-inducers. In the presence of the monosaccharides, vir genes are fully exploited at lower phenolic concentrations.

Stage 3: Initiation of T-DNA transfer

The bottom T-DNA strain is copied to make new single-stranded (ss) molecules by the activation of the *vir* genes system. The DNA fragments located in the middle of T-DNA borders will be integrated into the plant cells as single strand DNA. This step is taken by two proteins VirD1 and VirD2, they perceive the T-DNA border sequences and nicking the bottom strand at each border with the help of endonuclease enzyme. The nick position is considered as the starting and ending point for T-strand recovery. When the enzyme finish cutting, VirD2 covalently binds to the 5'-end of the ss-T-strand. This linkage ensures no disruption occurred by the exonucleolytic intervention to the 5'-end of the ss-T-strand (Dürrenberger *et al*., 1989) and differentiate the 5'-end as the termination site of the T-DNA transfer complex. VirD1 is necessary for the cleavage of supercoiled stranded substrate by VirD2 (Zupan and Zambryski, 1995; Christie *et al*., 1997).

Extensive studies on the two borders of T-DNA have indicated that mutation or deletion of the right T-DNA border results in complete loss of T-DNA transfer capacity, whereas only reducing transfer capacity when missing the left border (Hille *et al*., 1983). This explains that the T-strand synthesis is generated at the right border following by the 5' to 3' direction and the process still goes on regardless of the mutation or loss of the left border. So left border can act as an initiation point for ss-T-strand synthesis but with a lower in efficiency (Filichkin and Gelvin, 1993). The reason is stemmed from the presence of an enhancer sequence next to the right border (Peralta and Ream, 1985). This overdrive sequence is specifically

perceived by VirC1 protein (Toro *et al*., 1989). Deletion of *vir C* operon will weaken the virulence of the *Agrobacterium* strains

Stage 4: T-DNA transfer process. Two hypothesis for the T-DNA complex translocation.

The part that integrates into the plant cell is a ss-T-strand and the vehicle that transport it is a ss-T-strand protein complex. To incorporate to the plant nucleus, it has to pass through three membranes, the plant cell wall and cellular spaces. As widely accepted , the ss T-DNA-VirD2 complex is coated by the VirE2 protein, a single strand binding protein. This associated form eliminates the risk of nucleases attack and elongates the ss-T-strand, so it helps the translocation process through the membrane channel easier. However, this cooperative association does not support to immobilize T-DNA complex inside *Agrobacterium* (Zupan *et al*., 1996). VirE2 possesses two plant nuclear location signals (NLS) and VirD2 possesses one (Bravo Angel *et al.*, 1998). Therefore, both proteins contribute important parts in regulating the complex once incorporate into the nucleus of the plant cell.

 In translocation research, VirE1 was found to be in need for the export of VirE2 to the plant cell. However, other specific functions are still unknown (Binns *et al*., 1995). Bacteria that has VirE1 mutated is not able to export VirE2 which is inside the bacterium. These mutants that can be complemented when coinfection were taken place with a strain that can export VirE2, exhibiting that the VirE1 protein can be exported individually and the transmission event can happen without the transfer of VirE2 as part of the ss-T-DNA complex (Binns *et al*., 1995; Sundberg *et al*., 1996).

VirD4 proteins contributes in the translocation of T-DNA by generating the ATP-dependent linkage of protein complex. They are transmembrane proteins that crucially distribute at the cytoplasmic side of the cytoplasmic membrane (Okamoto *et al*., 1991).

VirB proteins functions similarly as other membrane-associated proteins (Kuldau *et al*., 1990). They generate of cell surface structure for the ssT-DNA complex transfer from bacterium to plant (Finberg *et al*., 1995). VirB4 and VirB11 are required to activate DNA transfer. VirB11 loses hydrophobic regions, forming the periplasmic domains. VirB4 specifies in association with the cytoplasmic membrane. These VirB4 are supposed to vary the shape of the ATP-dependent in the conjugation channel

because they compose of two putative extracellular domains that allow construction the shape of this protein. VirB4 and VirB11 are homodimers and heterodimers respectively (Dang and Christie, 1997). The VirB4 synthesis is highly associated with the generation and distribution of VirB3. Other protein, VirB7 is responsible for the conformation of the transfer system. VirB7 combines with VirB9 to form heterodimers complex. VirB9 is only accumulated stably when being in the heterodimer conformation showing that VirB9 alone may be unstable (Anderson *et al*., 1996). In this associated form, the monomeric subunits are linked by disulfide bridges. The VirB7-VirB9 complex is supposed to stabilize other vir proteins during the functionally assemble transmembrane channels (Fernandez *et al*., 1996; Spudich *et al*., 1996).

The presence of two accessory vir operons in the octopine Ti-plasmid, *vir*F and *vir*H support in conjugal transfer. The *vir*F operon only functions when the T-DNA complex is already inside the plant cells via the conjugal channel as for VirE2 export. *Vir*H operon encodes for the VirH1 and VirH2 proteins. These proteins are not essential but could make the transfer more efficient and detoxify certain plant compounds that might affect the bacterial proliferation (Kanemoto *et al*., 1989).

Stage 5: Integration of T-DNA into plant genome

Inside the plant cell, the ss-T-DNA complex is migrated to the nucleus passing through the nuclear membrane. VirD2 and VirE2 contributes important part in this step, besides VirF has a minor effect to this transfer (Hooykaas and Schilperoort, 1992). The ssT-DNA complex is up to 20 kb nucleoprotein comprising one 5'-end covalently attached VirD2 protein per complex. This complex is attached or coated by a large number of VirE2 molecules (approximately 600 per a 20 kb T-DNA), and each of them contains two nuclear location signals (NLS). The two NLS of VirE2 function in keeping the two sides of nuclear pores simultaneously open. The nuclear import is regulated by specific binding proteins which are present in cytoplasm.

The T-DNA strand is then pre-anneal with VirD2 and plant DNA. These combinations are less help in localization VirD2 for the ligation. The T strand and Plant DNA form a gap in 3'-5' strand of plant DNA. The plant DNA is then removed from its location, it is cut at the 3'-end position of the gap by endonucleases, and the first nucleotide of the 5' attaches to VirD2 pairs with a nucleotide in the top (5'-3') plant DNA strand. The 3' upper part of T-DNA and the strand that has been

removed are then digested away either by endonucleases or by 3'-5' exonucleases. After that, the 5' end which is attached to the VirD2 and other 3'-end of T-strand connects to the lower plant DNA strand. When the introduction is finished, a spiral is probably formed by a nick into the opposite plant DNA strand. This will activate the repair mechanism of the plant cell and complementary strand is produced following the early introduced T-DNA template strand (Tinland *et al*., 1995).

VirD2 contributes important part in the integration process of T-strand into the plant chromosome. When VirD2 releases, it supplies an energy source including in its phosphodiester bond, at the Tyr29 residue, with the first nucleotide of T-strand, giving the 5'-end of the T-strand for ligation to the plant DNA. This phosphodiester bond may act as electrophilic substrate for nucleophilic 3'-OH from nicked plant DNA (Jayaram, 1994).

After forming the ss-T-stand-VirD2 complex, this complex along with other Vir proteins are then exported into the host cell by a VirB4/D4 type IV secretion system. Once inside the host cell cytoplasm, the T-DNA is thought to exist as a mature T-complex , in which the entire length of the T-strand is coated with numerous VirE2 molecules. Participating in events within the host cell involving T-DNA cytoplasmic trafficking, nuclear targeting and integration into the host genome (VirD1, VirD2, VirE1, VirE2 and VirF). VirD2 and VirE2 may play role in targeting the T-strand to the nucleus. In addition, VirE2 likely protects T-strands from nucleolytic degradation in the plant cell. VirF may play a role in stripping proteins off the T-strand prior to T-DNA integration. Once transferred, the T-DNA is integrated into the plant genome, where the expression of some of the genes in the T-DNA allows production of phytohormones (auxin, cytokinin) and opines. The phytohormones are responsible for tumorous growth, by altering the hormone balance in the plant cell, this stimulates cell proliferation and gall formation. The ratio of auxin and cytokinin produced by the tumor genes determines the morphology of the tumor (root-like, shoot-like or dis-organized). Opines can be transported outside the plant cell and utilized by *A.tumefaciens* as a source of amino acids, carbons and nitrogens.

1.4.5 *Agrobacterium*-mediated transformation and achievements attained in the past

This natural vector system was widely used because protocol is easy to establish, cheap equipment and particularly the transgenic plants only obtained simple copy of insertions, including preferential integration of defined T-DNA into transcriptionally active regions of the chromosome (Hiei *et al*., 1997; Fang *et al*., 2002). Moreover, transgenic plants are generally fertile and foreign genes are often transmitted to progeny in a Mendelian manner (Rhodora and Thomas, 1996). This bacterial system has strong infection onto dicotyledonous plants but it is recalcitrant to monocotyledonous plants. However, some achievements has been already attained in the attempt to insert novel genes into monocots, for example rice, banana, corn, wheat, and sugarcane (Hiei *et al*., 1994; Cheng *et al*., 1997; May *et al*., 1995; Ishida *et al*., 1996; Enriquez-Obregon, 1998; Arencibia *et al*., 1998). To achieve with those monocots, superbinary vector was designed, it is a type of highly efficient vector designed with extra copies of the virulent (vir) genes or with mutations that increase the efficiency of vir genes. It has been achieved that a transferred large fragments (150 kb) into plant nuclear genome.

In dicots, *Agrobacterium*-mediated transformation is conducted using sterile leaf pieces, cotyledons, stem segments, callus suspension cultures and germinating seeds. Many factors should be considered to be suitable for the host plants as well as the bacterium in achievement of the transformation process. These factors are the use of feeder cells, modified *Agrobacterium* strains, infiltration of the bacteria, the period and temperature of co-cultivation. *Agrobacterium* is able to penetrate tobacco tissue through stomata opening after spraying with the bacteria (Escudero *et al*., 1997). Pre-wounding of sunflower shoot apices or banana meristematic tissue, cut from corm tissues or from shoot tips, with microprojectile or glass beads (Grayburn 1995) has shown the efficiency of pre-induction of bacteria vir genes (Schopke 1996). This explains that the invasion of *A. tumefaciens* will be easier when purposely enlarge the wounded site by pre-wounding the targeted plants. For some species, such as Arabidopsis thaliana, a floral dip method gives transgenic seeds without tissue culture requirement (Clough and Bent, 1998). Flowers are dipped in a culture of engineered Ti-plasmid and induce seed. Generally, approximately 0.1% of the seeds will contain the transgenes, these seeds are

recognized by their germinating ability on the medium containing the appropriate herbicide.

1.5 Binary vector and co-integrate vector.

1.5.1 Binary vector

A binary vector is a vector that includes the combination of a "disarmed" strain, which carries the Ti-plasmid without an original T-DNA, and an modified T-DNA within a plasmid. This could be expressed in both systems: *E.coli* and *A. tumefaciens*. The artificial T-DNA is often called a binary vector, now it's very popular in plant transformation

One of the early binary vector was constructed named pBIN19 (Bevan, 1984), and pBI121 was made shortly afterwards. Another popular vector was created named pPZP vectors (Hajdukiewicz *et al*., 1994) and pCAMBIA vectors (www.cambia.org), which are modfied from pPZP vectors. Recent advances attained in the binary vector system includes wide selection of cloning sites, high copy numbers in *E. coli*, high cloning capacity, improved compatibility with strains of choice, a wide pool of selectable markers for plants, and a high frequency of plant transformation. These vectors pBin19 and pPZP are very useful and popular among the plant scientists, a survey has been carried out and recorded as in the transformation of higher plant with the *A. tumefaciens* expression system, the derivatives of pBin19 were employed in 40% of the studies and derivatives of pPZP vectors in 30% of them.

The virulence genes that exhibit gene dosage effects (Jin *et al*., 1987) led to the development of a superbinary vector, which carried additional virulence genes (Komari, 1990).The development of a superbinary vector is based on virulence genes that exhibit gene dosage effects (Jin *et al*., 1987). Super binary vector is designed to carry additional virulence genes (Komari, 1990), they are highly efficient in the transformation of various recalcitrant plants, such as important cereals (Hiei *et al*., 1994; Ishida *et al*., 1996).

1.5.1.1 Basic structure of binary vectors

A binary vector consists of T-DNA and the vector backbone (Fig 1.6). T-DNA is divided by the two border sequences, the right border (RB) and the left border (LB), and may include multiple cloning sites, a selectable marker gene for plants, a reporter gene, selectable marker genes for the bacteria, plasmid mobilization be-

tween the bacteria and other genes of interest. The vector can be replicated in both *E. coli* and *A. tumefaciens* (Table 1.4).

Table 1.4 Binary vector and co-integrate (super binary) vectors (modified from Komori *et al*., 2007)

Vector	Plant selectable marker	Bacterial selection marker	Replication origin for *A.tumefaciens*	Replication origin for *E.coli*	Mobilization	Reference	Frequency of use in recent literature (%)
pBIN 19	Kan	Kan	IncP	IncP	Yes	Bevan, 1984	40%
pBI121	Kan	Kan	IncP	IncP	Yes	Jefferson 1987	40%
pCAMBIA series	Kan or hyg	Cm or Kan	pVS1	ColE1	Yes	www.cambia.org	30%
pPZP series	Kan or Gen	Cm or Kan	pVS1	ColE1	Yes	Hajdukiewicz *et al*., 1994	30%
pGreen series	Kan, hyg, sul, or bar	Kan	IncW	pUC	No	Hellens *et al*., 2000	3%
pGA482	Kan	Tc, Kan	IncP	ColE1	Yes	An *et al*., 1985	3%
pSB11	None	Sp	None	ColE1	Yes	Komari *et al*., 1996	3%
pSB1	None	Tc	IncP	ColE1	Yes	Komari *et al*., 1996	3%
pPCV001	Kan	Ap	IncP	ColE1	Yes	Koncz and Schell 1986	1%
pCLD04541	Kan	Tc, kan	IncP	IncP	Yes	Tao and Zang 1998	1%
pBIBAC series	Kan or hyg	Kan	pRi	Ffactor	Yes	Hamilton 1997	0%
pYLTAC series	Hyg or bar	Kan	pRi	Phage P1	No	Liu *et al*., 1999	0%

Kan, kanamycin; Hyg, hygromycin; Gen, gentamicin; Sul, sulfonylurea; Bar, phosphinothricin.   Cm, chloramphenicol; Sp, spectinomycin; Tc, tetracycline; Ap, ampicillin.

The RB and the LB are direct repeats of 25 bases and are the only cis-elements for the T-DNA transfer (Yadav *et al*., 1982). The RB and the LB are integrated in binary vectors as DNA fragments cloned from Ti plasmids, either octopine and nopaline type, pBin19 (Bevan, 1984), pPZP series (Hajdukiewicz *et al*., 1994), and pSB11 (Komari *et al*., 1996).
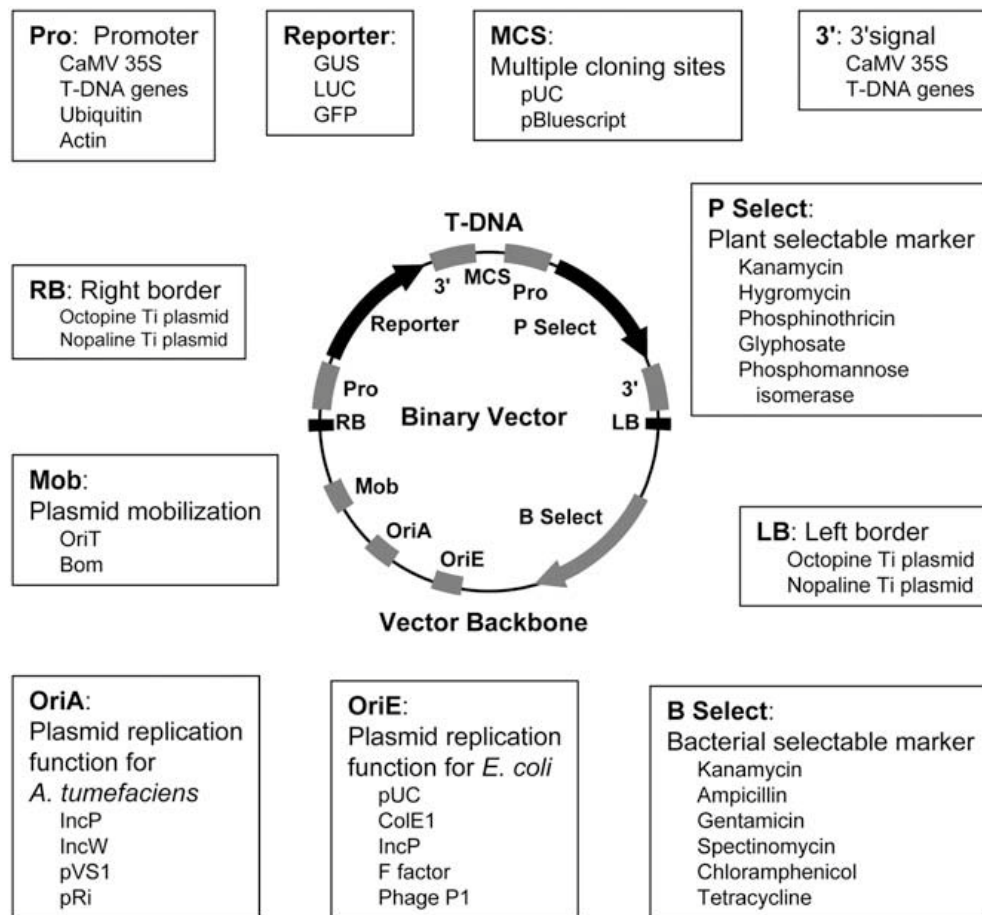
Figure 1.6 Key components of typical binary vector (Komori *et al*., 2007)

1.5.1.2 Selectable marker genes for plants

The selectable marker on the vector has capacity to resist the toxic substance when expressed on the transformed plant tissues. Genes that confer resistance to anti-biotics or herbicides, such as kanamycin, hygromycin, phosphinothricin, and glyphosate, are widely used. Kanamycin resistance is usually employed in the transformation of many dicotyledonous plants. Hygromycin resistance is the most effective in rice (*Oryza sativa*) transformation (Hiei *et al*., 1994), phosphinothricin resistance is the most effective in maize (Ishida *et al*., 1996). If creating herbicide-resistance plants, the selectable marker could be chosen to be the desired gene.

Selectable marker genes are driven by constitutive promoters. The promoters of the 35S transcript of Cauliflower Mosaic Virus (Odell *et al*., 1985) and the nopaline synthase of *A. tumefaciens* (Depicker *et al*., 1982) are very commonly-used in dicotyledons. The promoters of the ubiquitin gene of maize (Christensen *et al*., 1992) and the actin gene of rice are widely applied in monocotyledons (Zhang *et al*., 1991).

1.5.1.3 Reporter genes

The reporter genes are scored indicators of transformation. There are two types of reporter gene, a destructive and non-destructive assay system. The destructive one leads to the lethality of the transformed cells and the other type does not cause lethality. For example, *gus* or *luciferase* cause lethality for the transformed cells, whereas *gfp* is a non-destructive reporter gene that cause cell that express glow green under blue light.

Reporter genes connected to constitutive promoters may also be used to regulate the process of transformation. After the inoculation of plant cells with *A. tumefaciens*, in "transient expression," primary expression of the reporter genes is a good indication of transfer of the T-DNA from the bacteria to the nuclei of plant cells. Expression of the reporter genes later in a cluster of cells growing on selection media is an identification for integration of the T-DNA in plant chromosomes. A binary vector that carries a constitutive selectable marker and a constitutive reporter (Table 1.5) is very useful both in transformation experiments and in assays of gene expression.

Table 1.5 Plant cell reporter and selectable marker expression system (Glick *et al*., 2003)

Enzyme activity	Selectable marker	Reporter gene
Neomycin phosphotransferase	Yes	Yes
Hygromycin phosphotransferase	Yes	Yes
Dihydrofolate reductase	Yes	Yes
Chloramphenicol acetyltransferase	Yes	Yes
Gentamicin acetyltransferase	Yes	Yes
Nopaline synthase	No	Yes
Octopine synthase	No	Yes
β-glucuronidase	No	Yes
Streptomycin phosphotransferase	Yes	Yes
Bleomycin resistance	Yes	No
Firefly luciferase	No	Yes
Bacterial luciferase	No	Yes
Threonine dehydratase	Yes	Yes
Metallothionein II	Yes	Yes
Enol-pyruvylshikimate-3-phosphate synthase	Yes	No
Phosphinothricin acetyltransferase	Yes	Yes
β-galactosidase	No	Yes
Blasticidin S deaminase	Yes	Yes
Acetolactase synthase	Yes	No
Bromoxynil nitrilase	Yes	No
Green fluorescent protein	No	Yes

1.5.1.4 Multiple cloning site

Multiple cloning sites that are similar to those in pUC, pBluescript, and other standard vectors, are capable of inserting of gene of interest into appropriate locations of a binary vector. The "rare cutters" are the recognition site that have long recognition sequences serve as restriction enzymes because the DNA fragments that need to be inserted hardly to have this site. In some of the recently created vectors called modular vectors, a series of these rare sites are placed in the

T-DNA (Chung *et al*., 2005). There is a set of auxiliary plasmids, which have full sets or subsets of these rare sites, and some other restriction sites. Some of the plasmids also possess commonly used promoters, marker genes and 3' signal. In auxiliary plasmids, many constructed units may be built and then these units could be inserted into the modular binary vectors. Therefore, binary vector can be assembled by various types of expression cassettes.

1.5.1.5 Origin of plasmid replication

Origin of replication is the specific position on a DNA molecule where DNA replication begins. The copy number and the stability of the plasmids are determined by the types of replication . Those that carry the origin of replication of ColE1 are high-copy-number vectors. The origin of replication of pUC vectors gives a much higher copy number due to a mutation is a derivative of the origin of ColE1(Sambrook and Russell, 2001). Origin of IncP, IncW, or the F factor, are from low-copy-number vectors, which can be used for the transfer of large DNA fragments; for example, fragments larger than 15 kb.

1.5.1.6 Bacterial selection marker

In common cloning vectors, the antibiotic resistance genes which serve as bacterial selection marker can confer resistance to kanamycin, carbenicillin, gentamicin, spectinomycin, chloramphenicol, and tetracycline (Sambrook and Russell, 2001). In the medium that contains the specific antibiotic, the bacteria is still alive. This proves that the bacteria have already got the resistance genes which confer resistance to the antibiotic and carries the desired plasmids either. In some cases, it is necessary to be careful when using those. For example, after infection, the use of carbenicillin may not be effective to remove *A.tumefaciens* because the bacteria strain may carry a vector with an ampicillin resistance gene that can confer resistance to carbenicillin.

1.5.1.7 Plasmid mobilization functions

An origin of transfer (oriT) is a short nucleotide sequence approximately 500 bp of DNA that is required for transfer of a bacterial plasmid from a bacterial host to the target cell. Most binary vectors carry the origin of transfer (OriT) of IncP plasmids (Pansegrau *et al*., 1994) or the bom function of the ColE1 plasmid (Sambrook and Russell, 2001). Bom functions in association with the oriT to transfer a bacterial

plasmid from the host to the target cell. The plasmid with OriT or the bom may be transferred from *E. coli* to *A. tumefaciens* by a conjugal helper plasmid, such as pRK2013. This is not compulsory but a great advantage when vectors are incorporated into *A. tumefaciens* by electroporation because the conjugal transfer is such an efficient method.

1.5.2 Co-integrate vector

Co-integrate vector was invented with the aim of solving the problem that *Agrobacterium*-mediated transformation had been recalcitrant in monocotyledons previously. A co-integrate vector includes the 14.8-kb KpnI fragment that contains the virB, virG, and virC genes derived from pTiBo542, which is responsible for the supervirulence phenotype of an *A. tumefaciens* strain, A281 (Jin *et al*., 1987; Komari, 1990). The total size of the vector components is quite large, it is hard to insert desired genes into a co-integrate vector by ordinary methods. Therefore, through homologous recombination between an acceptor vector (co-integrate vector) such as pSN1 and an intermediate vector (recombination segment) such as pSB11 in the shared strain of *A. tumefaciens* can form an co-integrate vector (Fig 1.7). The integration forms a co-integrate plasmid that can be introduced into the plant cell via transformation.

The Ti plasmid has right border deleted and is substituted by the intermediate vector. The left border region remains as the cross over site with the intermediate vector. The intermediate vector carries the gene of interest and this intermediate vector has different restriction sites that make insertion of a variety cDNA. This intermediate vector also comprises the bacterial selectable marker genes. The vector possesses two regions of T-DNA, the nopaline synthase and the right border, the left border regulates recombination with a homologous region in the left border in the disarmed Ti-plasmid. By conjugation with *E.coli*, the co-integrate plasmid in then inserted into *A.tumefaciens* which contain the disarmed Ti plasmid.
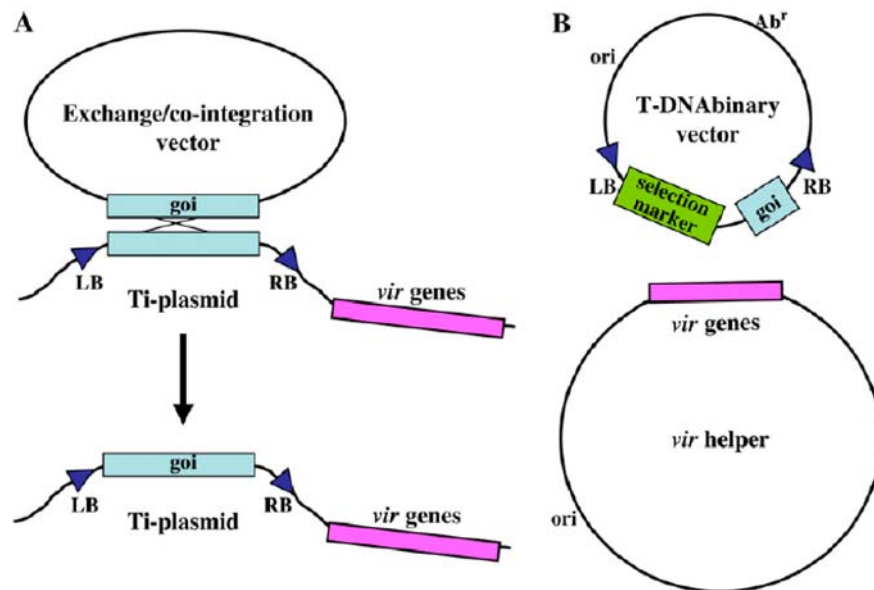
Figure 1.7 Co-integration/ exchange systems and binary vector systems to introduce genes into plants using *Agrobacterium* mediated-transformation A. Co-integrate vector system: an intermediate vector with T-DNA constructed in *E. coli* is transferred to an *A. tumefaciens* strain that carries an acceptor vector, and the co-integrate is created via homologous recombination between the shared sequences. Abbreviations LB, left border; RB, right border; goi, gene of interest; vir genes, virulence genes. B. Binary vector system, the T-DNA region contains the genes of interest. Vir proteins encoded by genes on a different replicon which transfer T-DNA from the bacterium to the host cell. The selection marker is used to identify successful plant transformation. ori, Origin of replication; Abr, antibiotic-resistance gene used to select for the presence of the T-DNA binary vector in *E. coli* (during the initial stages of gene construction) or in *Agrobacterium* (Lee *et al*., 2008)

The co-integrate vector comprises the disarmed Ti plasmid, intermediate vector and helper vector (vir helper). In the disarmed ti plasmid, the oncogenes in the T-DNA region is replaced by exogenous DNA. For the intermediate vector, the small (pBR322 based) plasmid with *E.coli* vectors have been used to eliminate the limit of large size of disarmed Ti-plasmids and the lack of unique restriction sites. This intermediate vector is replicated in *E.coli* and is transferred to *Agrobacterium* by conjugation. The vector is not able to replicate in *Agrobacterium*, so they carry DNA segments homologous to the disarmed T-DNA to form a co-integrate T-DNA system.

The vir helper is maintained in *E.coli* comprising transfer (tra) and mobilization (mob) genes that permit the transfer of the conjugation -intermediate vector into *Agrobacterium.*

CHAPTER II:

MATERIALS AND METHODS

2.1 Objectives of the experiment.

The experiment aims to conduct an experimental transformation based on *Agrobacterium*-mediated transformation on oil palm species (*Elaeis guineensis)*. This transformation method provides as an alternative choice for improving the traditional breeding and contributes for the research of making new traits expressed in oil palm cultivar. Immature embryos (IEs) were chosen as explant for tranformation. Gene β-glucuronidase (*gus*) is originated from an enzyme of *E.coli* and green fluorescence protein (*gfp*) isolated from the jelly fish *Aequoria victoria* were harbored by the two plasmids pCAMBIA 1304 and pCAMBIA 1302 respectively. These two novel genes will be integrated into the plant's genome through co-cultivation process that employed by the *Agrobacterium* as a biological vector carrying two separate plasmids. The results were obtained at least three days after transformation and were observed under dissecting microscope for confirmation.

2.2 Prepare materials for co-cultivation.

2.2.1 Prepare explants for co-cultivation

Oil palm (*Elaeis guineensis* Jacq. var. Tenera) fruit bunch 9-12 weeks (Fig 2.1) old were obtained from Advanced Agriecological Research Center (AAR)

Figure 2.1 Fruit bunch of *Elaeis guineensis* Jacq. var. Tenera 12 weeks old.

Oil palm fruits were detached from the fruit bunch using prunning scissors (Fig 2.2). They were then washed with normal tap water several times until clean. Subsequently, the fruits were soaked with distilled water added with a few drops of Tween 20 for 20 minutes. After 20 minutes, the fruits were rinsed in water and thereafter soaked in absolute ethanol for 15 minutes prior to excision.

Figure 2.2 Loosen oil palm fruits.

The oil palm fruits were excised in the laminar flow, and cut horizontally (Fig 2.3a). Sterile scalpels were used to excise the immature embryos (IEs) and the IEs were then plated on N_6O medium (Fig 2.3b)
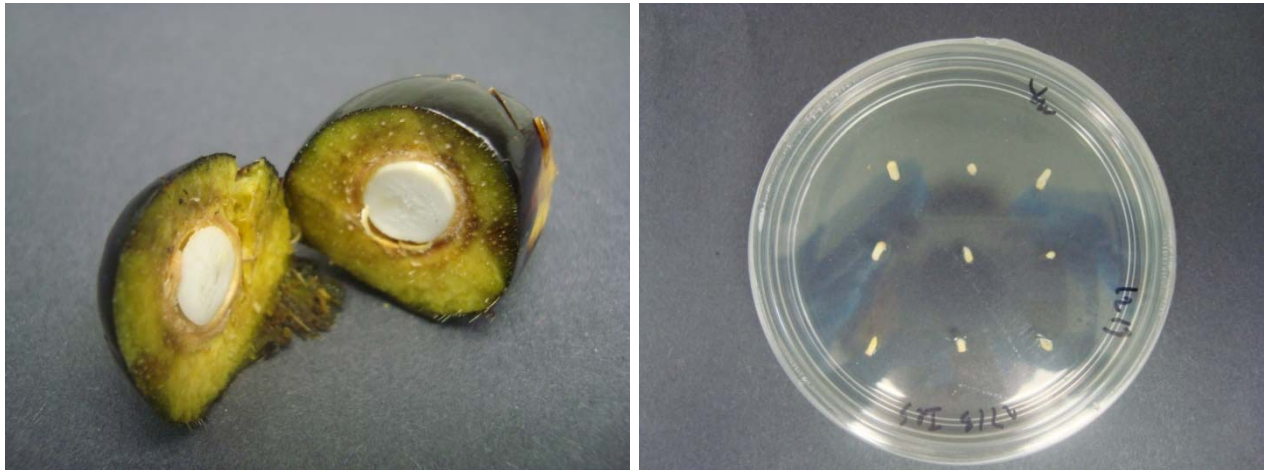
a) b)

Figure 2.3 Excision and plating IEs on N_6O medium. a) Horizontal cutting of oil palm fruit. b) Excised IEs plated on N_6O medium.

2.2.2 Preparation of the N_6O medium

The N_6O medium (Chu et al., 1975) is a mix of several components including: macronutrients, micronutrients, vitamins and sucrose. Table 2.1 refers to the stock solution containing necessary components of this cultured medium.

Table 2.1 Stock solution preparation of N_6O medium

	Composition	Concentration (mg/l)	Volume from stock (ml)
Macronutrients	$CaCl_2.2H_2O$	1660	100
	$(NH4)_2SO4$	4630	100
	KNO_3	28300	100
	KH_2PO_4	4000	100
	$MgSO_4.7H_2O$	1850	100
Micronutrients	KI	83	10
	H_3BO_3	160	10
	$MnSO_4.4H_2O$	440	10
	$ZnSO_4.7H_2 0$	150	10
	$FeSO_4.7H_2O$	2785	10
	Na_2EDTA	3725	10
Vitamins	Myo-inositol	10	10
	Nicotinic acid	1	1
	Pyridoxine-HCl	1	0.5
	Thiamine-HCl	1	0.5

The ingredients were mixed into the 1L beaker 1000 ml with the designated volumes according to the volume from stock listed in the Table 2.1 , 30 g sucrose were then added and the pH of the solution reached to 5.8.

### 2.2.3 Preparation of media for *E.coli* and *Agrobacterium* LB, LB + 5mM Glucose (LB-G).

LB broth solution was prepared first. The ingredients included 10g bactotryptone, 5g bactoyeast and 10g NaCl. The solution was then stirred till all the components were dissolved. This LB was used for *E.coli* culture medium, LB- 5mM Glucose (LB-G) would be used for *Agrobacterium tumefaciens*. LB-G was prepared with the ingredients of LB and the solution was then added with 5mM glucose. The pH of LB broth should be 7. For the medium, 15g bactoagar was added into the mix solution and then the solution was autoclaved, and poured into petri dishes. From the LB medium petri dish, *E.coli* strain TOP 10 (commercial strain similar like DH10B) were streaked on the surface of the agar to get the single colonies for further use, this

was done in the laminar flow. Similarly, in the LB-G medium petri dish, dishes were prepared containing single colonies of *A.tumefaciens* that have those plasmids pCAMBIA 1302 and pCAMBIA 1304.
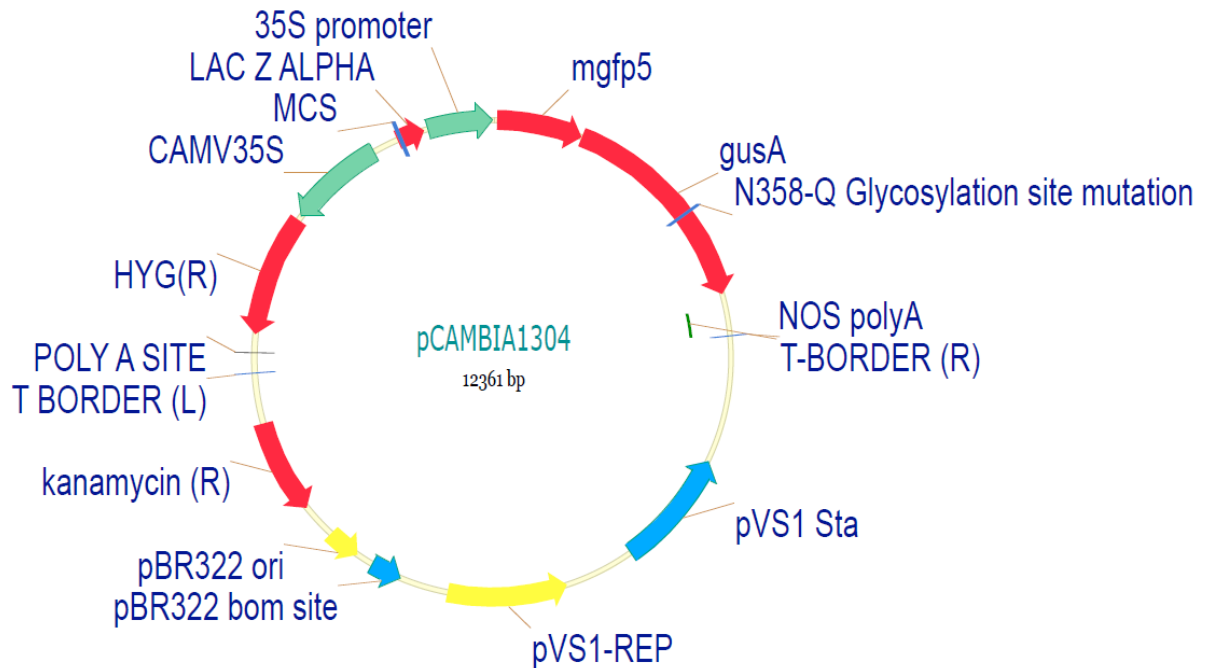
2.3 Plasmid confirmation

2.3.1 Plasmid Extraction

E.coli was cultured in the LB medium + 50 g/l kanamycin prior to plasmid extraction. *E.coli* strain TOP 10 was used, it harbored two separate plasmids (pCAMBIA 1302 and pCAMBIA 1304). Maps of these two plasmids were shown in Figure 2.4.
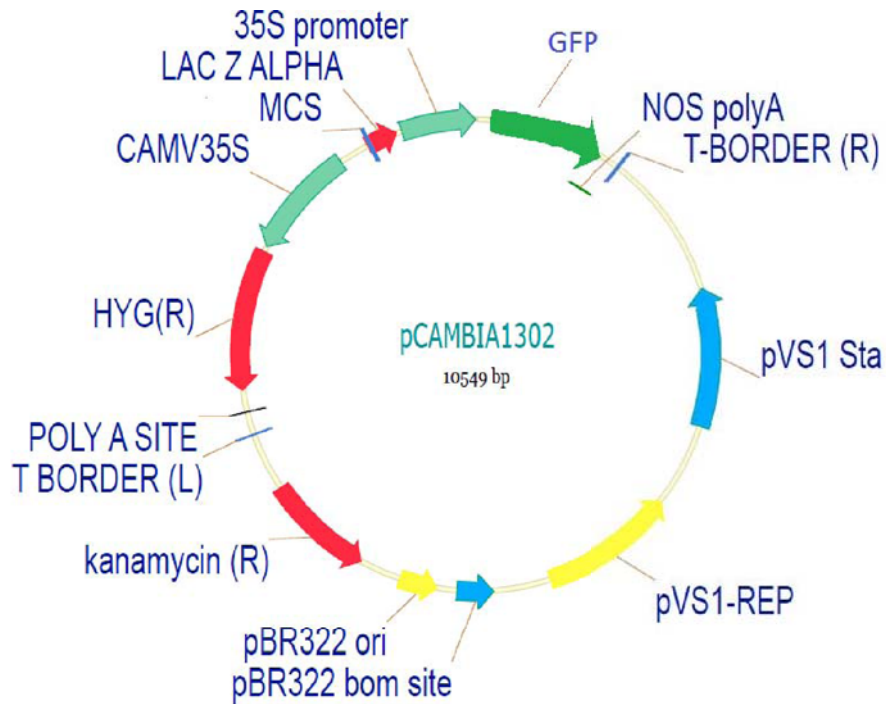
a) Plasmid pCAMBIA 1302 containing the green fluorescence protein (GFP).

b) Plasmid pCAMBIA 1304 containing the *gusA* gene.

Figure 2.4 Schematic diagram of plasmid pCAMBIA 1302 and pCAMBIA 1304.

Plasmid extraction based on the alkaline lysis method (Birboin and Doly, 1979) was employed. For the material used for experiment, 3 solutions were prepared as follows: Solution I contained 50mM glucose, 25 mM Tris-HCl (pH 8), 10mM EDTA (pH 8), was autoclaved and stored at 4°C. Solution II contained 0.2 M NaOH, 1% (w/v) SDS (sodium dodecyl sulphate), this solution was stored at room temperature. Solution III contained 60 ml of 5M potassium acetate, 11.5 ml acetic acid, 28.5 ml water, this solution was then autoclaved and kept on ice before used.

Solution I contained glucose that supplied the osmotic shock that led to the breach of cell wall and membrane. EDTA functioned in inhibits nucleases. Solution II contained NaOH and SDS to lyses the cell completely, the alkaline pH only denatured the chromosome DNA but not the covalently closed circular plasmid DNA. Solution III contained potassium acetate and glacia acetic acid was for neutralize the alkaline pH. It included precipitation of protein and formed of SDS-protein complex, chromosomal DNA also could be denatured and aggregated with protein.

The plasmid extraction is a multiple-step process. First, *E.coli* TOP 10 suspension was pelleted in each eppendorf tube by centrifuging at 12 revolution per minute (rpm) within 3 minutes, then the supernatant was removed. Second, the pellet was

refilled with 100 µl prechilled solution I, then vortexed. Eppendorf tubes were then incubated within 5 minutes in room temperature. Third, 200 µl solution II was added and the tubes were inverted gently from 2 to 3 times, then incubated on ice for 5 minutes. Next, 150 µl pre-chilled solution III was added, then the tubes were inverted gently 4-5 times. These tubes were then incubated on ice for 5 minutes. They would be then centrifuged at 13000 rpm for 15 minutes, the reason for centrifuging was to pellet the protein and DNA to aggregate to each other. After that, the supernatants were transferred into new tubes, these supernatants were what would be continued to do with because plasmids were present in here, so all of the upper parts were carefully transferred into new tubes. Next, 1 volume of phenol-chloroform-isoamyl alcohol was added and the new tubes were mixed well by inversion, then centrifuged at 13000 rpm within 5 minutes. Later, the top phases were pipetted into four new eppendorf tubes, and the each tube was then added with 2 volume of 100% ethanol, the reason alcohol was used in this step was to precipitate the plasmid. New tubes were left in the room temperature around 2 minutes. Next, these tubes were centrifuged at 13000 rpm within 5 minutes, then these plasmids were present in the pellet. After that, the supernatants were removed, the pellet was then washed with 1ml of 70% pre-chilled ethanol. The pellet was incubated at -20°C overnight. Next day, eppendorf tubes were centrifuged at 11000 rpm within 20 minutes to get the plasmid precipitation. The supernatant was removed and these tubes were made air-dried. Final step was to resuspend the pellet in 30 µl TE buffer and the tubes were stored at -20°C for verification later.

2.3.2 Plasmid confirmation

Heat shock method was employed to transfer the plasmid pCAMBIA 1302 and pCAMBIA 1304 from *E.coli* TOP 10 to *Agrobacterium tumefaciens* strain LBA 4404. The heat pulses helped to release of lipids and opened the pores on the cell surface, which allowed DNA to migrate from the host to the target cells. It also lowered the membrane potential, which made the plasmid transfer easier. Placing the cells on ice after the shock made the pores close so that it prevented the plasmid to escape.

E.coli competence cells in CaCl$_2$ were thawn and mixed with 1 µl plasmid pCAMBIA 1302 and 1304 respectively. The tubes were then incubated on ice for 30 minutes and then exposed to heat when incubated in the water bath 42°C within 1 minute. After that, these tubes were incubated on ice again for 2 minutes. SOC solution

(20g bacto-tryptone, 5g bactoyeast, 0.5g NaCl, 1M KCl) was added immediatedly into the tubes after incubating on ice. Subsequently, the tubes were incubated at room temperature for 1 hour and were then centrifuged 13000 rpm within 2 minutes. The supernatants were discarded and the pellets were streaked into plates LB + 50 g/l kanamycin.

The restriction enzymes *Bgl* II and *Eco*RV were performed on plasmid pCAMBIA 1302 and *Bgl* II and *Bam*HI were performed on plasmid pCAMBIA 1304. Table 2.2 list the advised amounts of components in the restriction enzyme digestion. Four eppendorf tubes were used for the restriction enzyme digestion, the components were put in this order (from first to last): water, buffer, enzyme and DNA. These eppendorf tubes were then incubated in water bath 37^0C one and a half hour for enzyme digestion.

Table 2.2 Components of restriction enzyme digestion for both pCAMBIA 1302 and pCAMBIA 1304.

	Water (µl)	Buffer (µl)	Enzyme (µl)	Plasmid DNA (pCAMBIA 1304) and (pCAMBIA 1302) (µl)
Bgl II	15	2	0.5	3
Bam HI	15	2	0.5	3
*Eco*RV	15	2	0.5	5

Electrophoresis was conducted with 0.8% (w/v) agarose. Agarose gel 30 ml was prepared. DNA reaction mix contained 5 µl sample DNA, 1 µl loading dye and 4 µl distilled water to verify the concentration of the plasmid. Both plasmids were applied the same amount of DNA reaction mix. λ-*Hind*III marker was used as the ladder, the DNA reaction mix and the restriction enzyme mix were slowly pipetted into the wells of the gel, the restriction enzymes would cut plasmid at specific locations (Fig 2.5) with the expected fragments according to the Table 2.3.
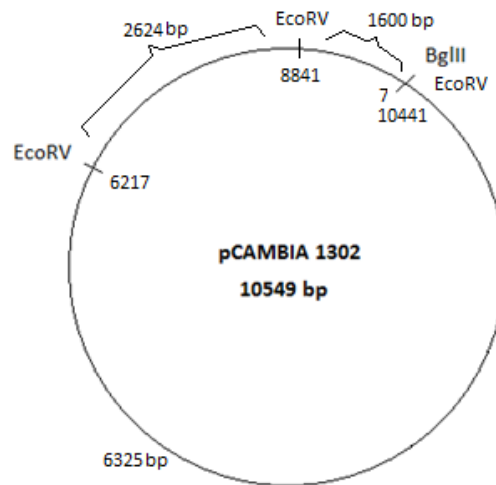
a) Map of restriction enzyme digestion for pCAMBIA 1302. The numbers inside the circle represent the digested positions of the respective restriction enzymes. *Bgl* II digested at 7. *Eco*RV digested at three different positions 10441, 8841 and 6217. The numbers outside the circle represent the expected fragments (Table 2.3).
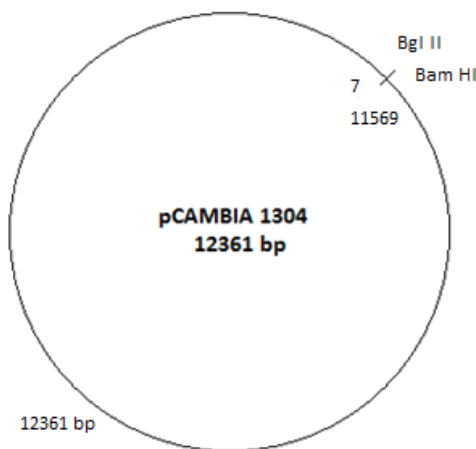
b) Map of restriction enzyme digestion for pCAMBIA 1304. The numbers inside the circle represent the digested positions of the respective restriction enzymes. *Bgl* II digested at 7. *Bam* HI digested at 11569. The numbers outside the circle represent the expected fragments (Table 2.3).

Figure 2.5   Schematic diagram of restriction enzyme digestion for plasmids pCAMBIA 1302 and pCAMBIA 1304.

Table 2.3 Sizes of the fragments digested by the restriction enzymes for plasmid DNA (pCAMBIA 1304) and (pCAMBIA 1302)

Restriction enzyme	Expected fragment(s) (bp)	
	pCAMBIA 1302	pCAMBIA 1304
Bgl II	10549	12361
Bam HI		12361
EcoRV	1600, 2624, 6325	

2.4 Co-cultivation process using *Agrobacterium tumefaciens*

2.4.1 Prepare the tubes for co-cultivation

The single colonies were taken from petri dishes. Falcon tubes were prepared containing the ingredients as follows:

	Plasmid from *A.tumefaciens*		
Environment	pCAMBIA 1302	pCAMBIA 1304	pCAMBIA 1302 + pCAMBIA 1304 (1:1)
LB-G (20 ml +10 µl Kanamycin)	Tube 1	Tube 2	Tube 3

In these tubes, 20 ml of LB-G and 10 µl Kanamycin (50 µg/ml) were dispensed into each tube. Single colonies from pCAMBIA 1302 and 1304 were taken to put into those falcon tubes respectively. Falcon tubes were put into the incubator for shaking overnight. The machine was adjusted 200 rpm and the temperature was set at 28^0C. The time for shaking was 17 hours, it was enough time for the bacteria to growth up to acceptable concentration.

2.4.2 Transformation

Single colonies from pCAMBIA 1302 and 1304 were taken to put into falcon tubes respectively. These falcon tubes were then shaken with 200 rpm at 28^oC, the OD_{600} was measured after each hour till reaching the desired OD_{600} and the bacteria density was 10^8, the co-cultivation was started. In the laminar flow, the IEs were immersed in the solution prepared in the falcon tubes, the IEs were carefully taken

out from the N_6O media by sterile scalpels to transferred into the falcon tubes. Each falcon tube contained 27 IEs and was then shaken within 30 minutes. After 30 minutes, the IEs were blotted dry on whatman filter paper and left for drying within another 30 minutes. These IEs were then transferred to new N_6O media, labeled carefully. Co-cultivation was cariried out in the dark for one day and temperature of the cultured room is 25^oC. IEs were co-cultivation in the dark because the IEs after *Agrobacterium* infecton were under stress due to the integration of T-DNA from the bacteria to the plant cells, so for reducing the stress transformed IEs were safely covered with aluminum foils.

2.4.3 Cefotaxime wash

One day after co-cultivation, IEs were taken out from the medium and put into blank petri dishes. 27 IEs of one group (each group was infected with *Agrobacterium* harboring different plasmid 1302, 1304 and 1302 + 1304) were put into one petri dish for washing them with cefotaxime (250 mg/l). The washing period was 30 minutes, these IEs were dipped into the solution. After 30 minutes, these IEs were blotted dry by leaving them on the filter paper. When being completely dry, they were transferred into new N_6O media. After that, they were put back at 25^oC in cultured room and left there about 3-5 days prior to examining the results.

2.4.4 GUS assay

For the GUS staining solution, the components were prepared as follows 0.5 mg/ml X-Gluc in Dimethyl Formamide, 0.1% Triton X-100 in 50 mM Phosphate buffer pH 7.2. The IEs were put into the eppendorf tubes and the tubes were dispensed with 800 μl GUS staining solution (X-Gluc), these tubes were then incubated in the water bath 37^oC and left overnight. The samples were left 18 hours in the water bath before observing the results.

CHAPTER III:

RESULTS

3.1 Plasmid verification

The plasmid pCAMBIA 1302 and pCAMBIA 1304 were extracted using alkaline lysis method. The concentrations of plasmid DNAs were obtained using the nano drop measurement at 240 nm one day after plasmid extraction was carried out. The result is illustrated in table 3.1

Table 3.1 Concentration of plasmid DNA after extraction.

Plasmid	Concentration (ng/µl)
pCAMBIA 1302	
Sample 1	87.8
Sample 2	103.2
Sample 3	71.3
Sample 4	76.8
pCAMBIA 1304	
Sample 1	102.9
Sample 2	348
Sample 3	2671
Sample 4	175

The sample which obtained the highest concentration in each group was selected to do the plasmid verification. So the result of gel electrophoresis was from sample 2 of pCAMBIA 1302 and sample 3 of pCAMBIA 1304 (Fig 3.1). The restriction enzymes *Bgl* II and *Eco*RV were performed on plasmid pCAMBIA 1302 and *Bgl* II and *Bam*HI were performed on plasmid pCAMBIA 1304. The reason to do the plasmid verification was to confirm the presence of the plasmid DNAs that were harbored by *Agrobacterium.* In addition, it was to make sure that the plasmid pCAMBIA 1302 carried the *gfp* gene and pCAMBIA 1304 carried the *gus* (*uidA*). From the Table 3.1,

there were different concentrations of plasmid yield, it was due to the pellet centrifuge from the same *E.coli* suspension. If the pellet yield was high then would give a high amount of plasmid concentration and vice versa.
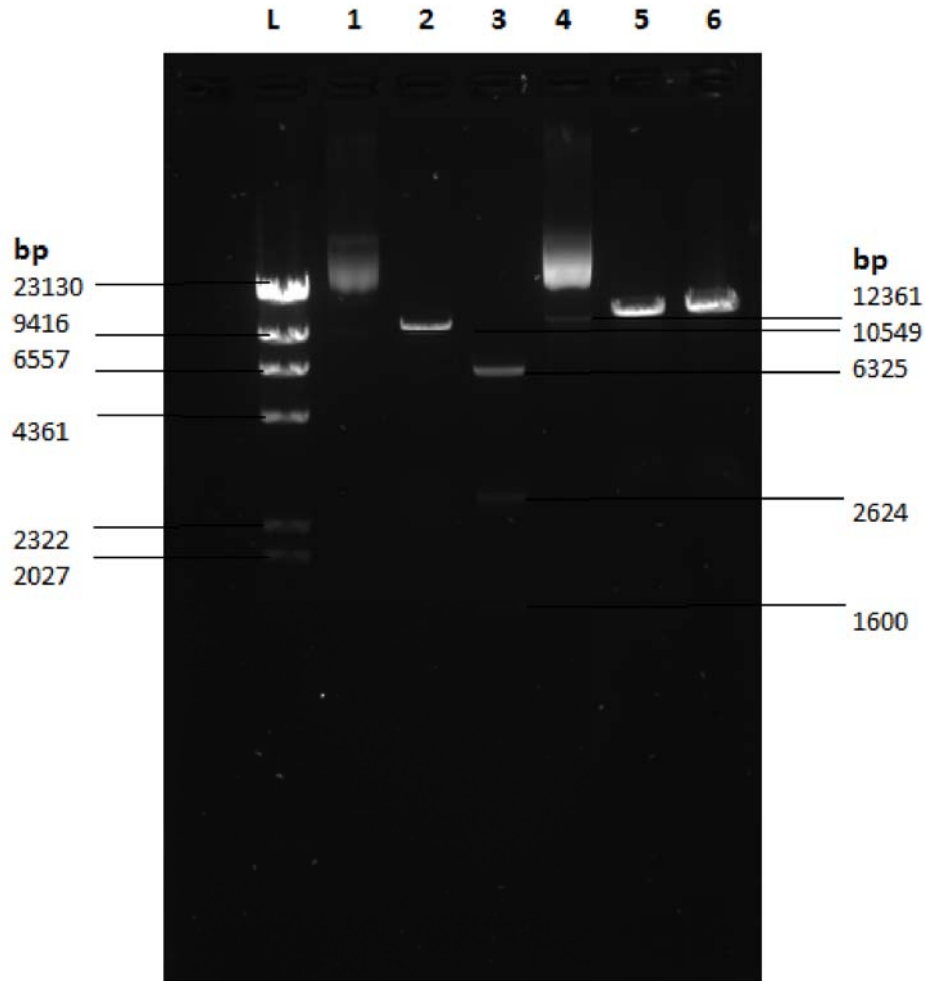

Figure 3.1 Gel electrophoresis result for detection of plasmid DNAs and the present of the *gus* and *gfp* gene. L: λ-*Hind* IIIladder. Lane 1: pCAMBIA 1302 (10549 bp) Lane 2: pCAMBIA 1302 digested with *Bgl* II (10549 bp). Lane 3: pCAMBIA 1302 digested with *Eco*RV (6325, 2624, 1600 bp). Lane 4: pCAMBIA 1304 (12361bp). Lane 5: pCAMBIA 1304 digested with *Bgl* II (12361 bp). Lane 6: pCAMBIA 1304 digested with *Bam* HI (12361 bp).

3.2 Growth curve of *A.tumefaciens*

Two conical flasks were added with 25 ml LB-G and 50g/l kanamycin (km). Each conical flasks contained *Agrobacterium* harboring pCAMBIA 1302 and 1304 respectively. A single well-isolated colony from a recently subcultured LB agar plate was taken to put into each conical flask. The density of the bacteria was recorded after each hour for measurement of the OD_{600} (Fig 3.2)
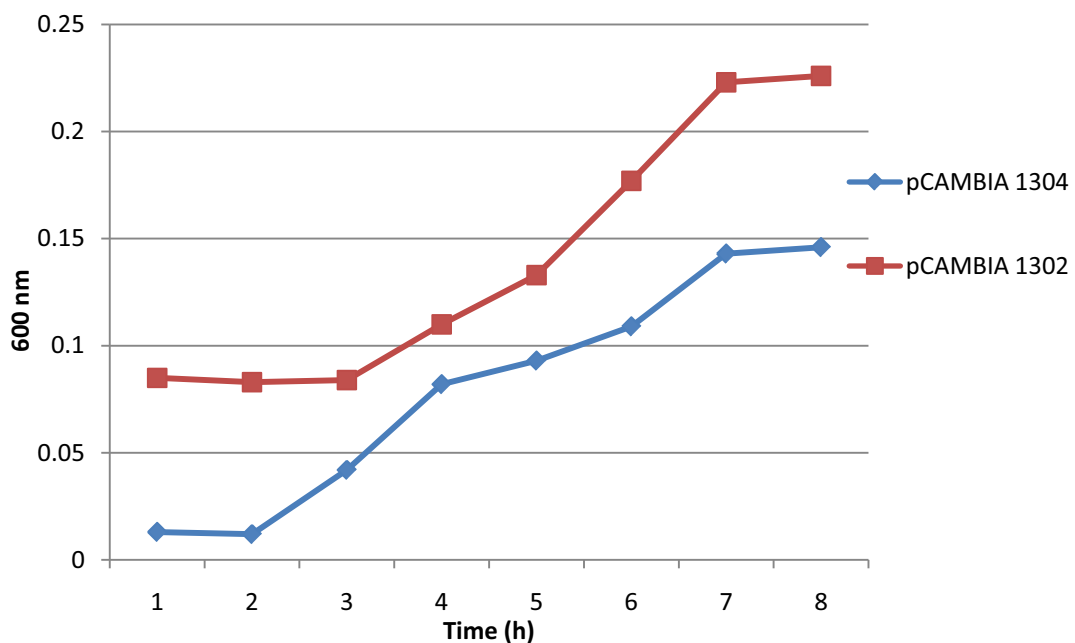

Figure 3.2 Growth curve of *Agrobacterium* harboring pCAMBIA 1302 and 1304 respectively.

At the beginning, the density of bacteria were recorded and were measured after each hour, the result was shown in the Table 3.2. At first, the bacteria may grow slowly, but later at the log phase that started from the fourth-hour, the density was gradually increased, the bacteria proliferated faster than in the log phase.

Table 3.2 OD_{600} of *Agrobacterium* strain LBA 4404 harboring pCAMBIA 1302 and pCAMBIA 1304 measured within 8 hours.

Time	pCAMBIA 1302	pCAMBIA 1304
1	0.085	0.013
2	0.083	0.012
3	0.084	0.042
4	0.11	0.082
5	0.133	0.093
6	0.177	0.109
7	0.223	0.143
8	0.226	0.146

Two LB-G tubes containing the bacteria harboring pCAMBIA 1302 and pCAMBIA 1304 respectively, and the third tube contained the mix of pCAMBIA 1302 + pCAMBIA 1304 with the ratio 1:1 were put into the incubator shaker within 17 hours through the night. The fourth hour was the duration of the log phase when the bacteria grew actively, the log phase endured 13 hours later and when it came to 18 to 19 hours, the bacteria stopped multiplying and started dying gradually as the OD_{600} reduced and reached minus number (data not shown). Therefore, the concentration along with the active state were achieved the most in the last hour of the log phase (17 hours), the density reached 1.9 for the 1304, 1.8 for 1302 and 1 for the combination of 1302+1304. Based on the OD_{600}, the concentration of bacteria was calculated followed the McFarland Standard (Table 3.3).

Table 3.3 The OD_{600} attained at the seventeenth hour (17 hours) of *A.tumefaciens.*

	1302	1304	1302+1304
Optical density (OD_{600})	1.8	1.9	1
Concentration of the bacteria (bacteria/ml)	41×10^8	45×10^8	19×10^8

3.3 Histochemical GUS assay

The histochemical GUS assay was employed to determine the success of the transformation events in immature embryos (IEs). In single transformation, the transformed IEs showed strong GUS activity around their bodies and smears were observed very randomly distributed around the transformed IEs. On the day 7th after co-cultivation, the IEs were exposed with blue stripes along the bodies of the embryos. On the day 15th after co-cultivation, GUS activity was exhibited more intensively in IEs in single transformation (Fig 3.3), which indicated that the T-DNA region from the pCAMBIA 1304 construct had been transferred into the genomes of the IEs. In co-transformation, the number of transformed IEs was achieved less than single transformation, the GUS activity was observed concentrated at some regions in the IEs (Fig 3.4)

The GUS activity expresses in different levels because of two factors. One is that the transgenes may be integrated into the genome at different positions, the other factor is different copy numbers of the transgenes could have been inserted into the genome (Saini *et al*., 2003). For the transformed tissues that did not express GUS activity, it may be the cause of instability of the transgenes, inefficient transgene integration or gene silencing. In contrast, untransformed IEs which served as controls did not exhibit any blue coloration.
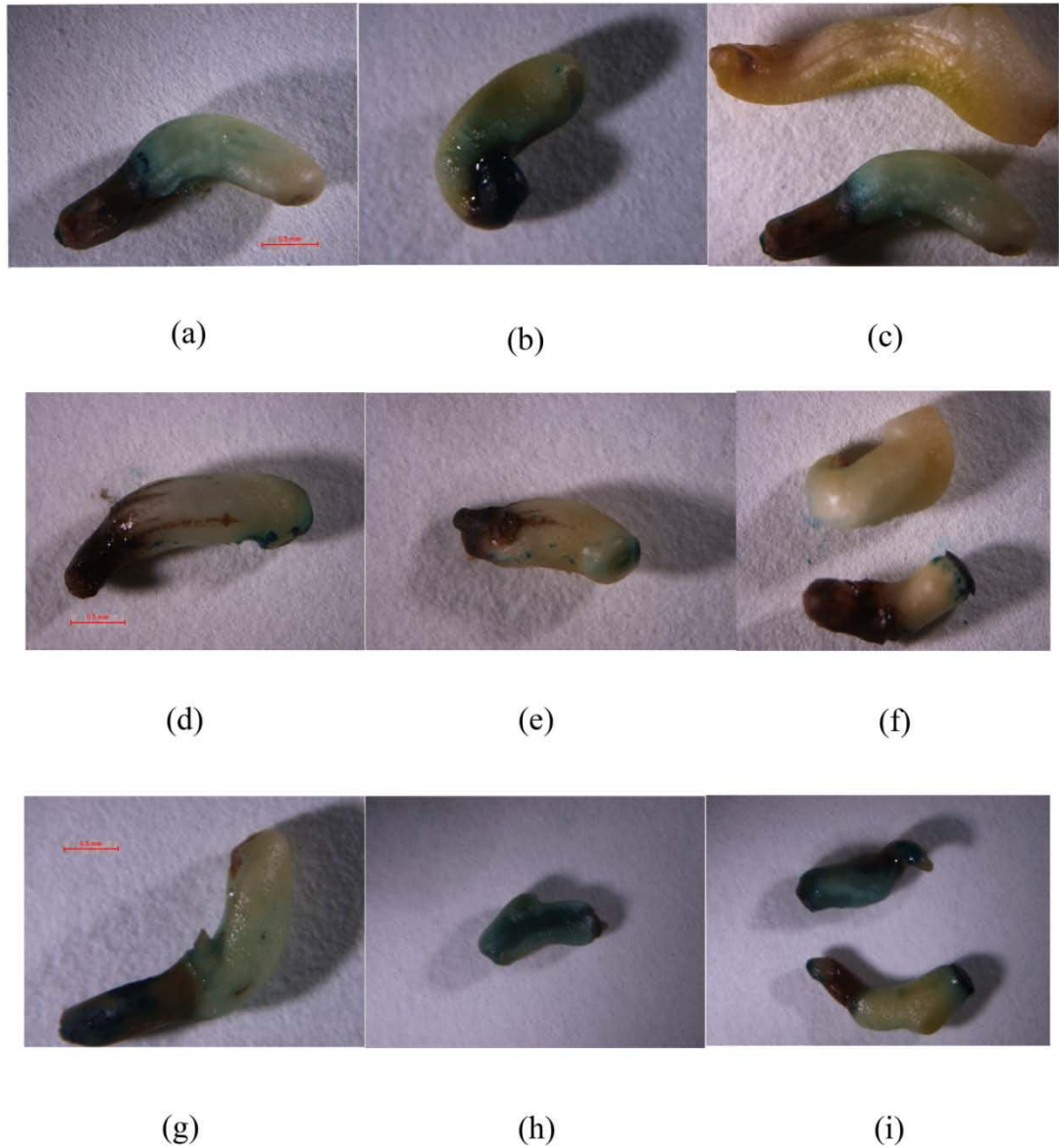
Figure 3.3 Different levels and expression patterns of the *gus* gene in the putatively transformed IEs. Transient expression on day 7th after co-cultivation (a-b), transformed IE was compared with the control on the day 7th (c). On day 12th blue spots distributed randomly around the IEs (d-e), transformed IE exposed blue dots compared to the negative control (f). On day 15th GUS activity expressed extensively all over the surfaces of some IEs (g-i). Scale bar: 0.5 mm

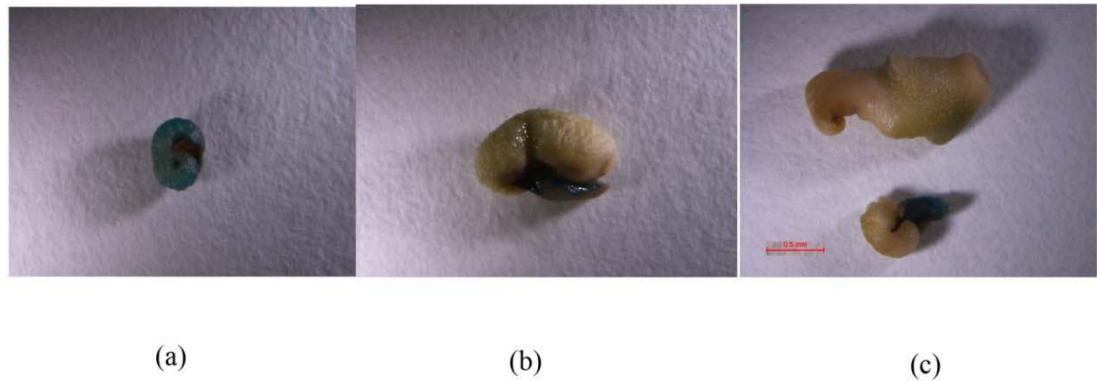(a)   (b)   (c)

Figure 3.4 In co-transformation, transformed IEs were subjected to GUS assay 18 days after co-cultivation (a-b), transformed IEs were compared with the control on the day 18th (c). Scale bar: 0.5 mm

The transformed IEs showed a lower level of growth and development in comparison with the controlled IEs. This explained the transformed IEs were under stress and the activity of *Agrobacterium* suppressed the growth of the IEs. In addition, the brown regions were observed in the bodies of the IEs showed that the phenolic compounds (acetosyringone) were excreted excessively from the reactions of the IEs to the infection of the bacteria. It was the hypersensitive response of the plant tissues that stop the invasion of the bacteria for preventing further loss of nutrients to the *Agrobacterium*. For reducing the browning activity, the concentration of the bacteria should be more considered. The untransformed IEs can reach approximately 12 mm to 14 mm 3 weeks after placing them on the N_6O medium (Fig 3.5) and the transformed IEs only reached 7-9 mm at the same time. Therefore, the transformed tissues always grow slower and less developed compared to the untransformed IEs.
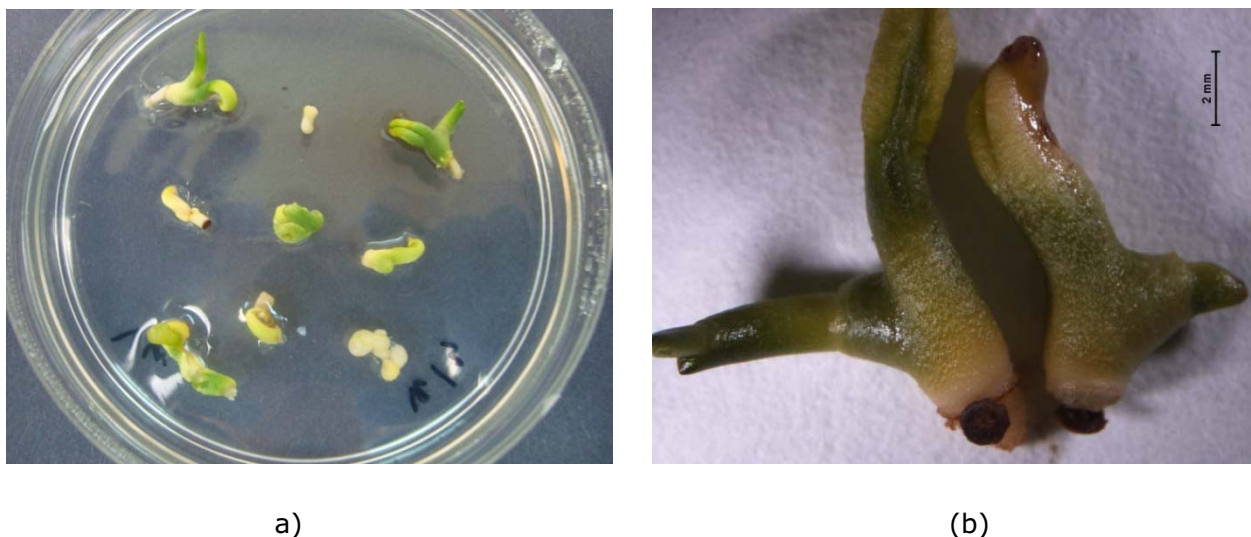
<div align="center">a)   (b)</div>

Figure 3.5 Untransformed IEs as a negative control. (a) untransformed IEs grew up to 12 mm to 14 mm 3 weeks after placing them on the N_6O medium. (b) Amplification of untransformed IEs after 3 weeks growth.

The transformation frequency was calculated based on the number of IEs that exhibited GUS activity. The IEs transformed in co-transformation showed approximately 17.39% transformation frequency, which is less than the IEs transformed in single transformation 64%. The IEs in co-transformation exhibited lower expression of the *gus* gene because they were suppressed by one another activity. Two plasmids with two different genes would interact to each other and one may suppress the other, the result was lower expression of GUS activity compared to single expression and no Green Fluorescence Protein (GFP) was found in the co-transformed tissues at the end.

3.4 Analysis of GFP activity

The IEs were observed under blue light of the Nikon dissecting microscope 4 days after transformation. In single transformation, transformed IEs were obtained with the transformation efficiency 41.67%. The IEs luminesced in some regions that we called them chimeric (Fig 3.6). In co-transformation, there were no GFP activity demonstrated. Vector sequences that originate from the bacteria could trigger gene silencing because the foreign sequences are unable to bind eukaryotic nuclear proteins (Sandhu and Altpeter, 2008). In this case, the gene from bacteria was unable to be expressed because of the gene silencing. However, the transformation

efficiency is quite high in single transformation both in GFP and in GUS assay compared to co-transformation (Table 3.4).

Table 3.4 Transformation frequency calculated based on the successful transformed IEs from single- and co-transformation.

Treatment	Number of transformants	Number of IEs survival after inoculation	Positive GUS	Positive GFP	Transformation Efficiency (%)
Single transformation Gus assay	27	25	16		64
Single transformation GFP activity	27	24		10	41.67
Co-transformation Gus + GFP	27	23	4		17.39

Figure 3.6 GFP expression of the transformed IEs. Transformed IEs of single transformation were observed 4 days after inoculation; the chimeric IEs exhibited GFP expression in some regions (a-f); the untransformed IEs served as the controls (g-h).

CHAPTER IV:

DISCUSSION

4.1 Single transformation and co-transformation

In my experiment, single transformation achieved higher transformation efficiency than co-transformation. The single transformation for GUS and GFP was 64% and 41.67% respectively while the co-transformation only attained 17.39%. One of the reasons is, in co-transformation, the expression of one gene suppressed the expression of another, this is known as gene silencing. When there are two multiple foreign genes that integrate into plant genome, one is suppressed by another that cause the degradation of specific mRNAs, the degradation was sequence-specific and resulted in the silencing of one foreign gene (Slater *et al*., 2008). Therefore, *gfp* genes in the transformed IEs were degraded in co-cultivation so that they did not express at all.

In the study of Abdullah (2005), oil palm was successful transformed with both *cry1A*(B) gene (pCAMBt1) and *CpTI* gene (pMRT1301) with an overall frequency of about 20%. The *Agrobacterium* strain LBA4404 was used, it harbored different plasmid LBA4404 (pCAMBt1) and (pMRT1301). Further study showed that the frequency of co -transformation of oil palm tissues is only a half of those occurred in single transformation.

Komari *et al*., 1996 reported that in transformation of tobacco employed by *Agrobacterium*-mediated transformation, the transformation frequency of single transformation is twice as much as co-transformation. In rice transformation, single transformation was even third or fourth times higher than co-transformation (Table 4.1)

Table 4.1 GUS expression in the drug-resistant transformants (Modified from Komari *et al*., 1996)

| Plant | Strains transformed | Number of transformants | | |
		Total	GUS positive	%
Tobacco	LBA4404 (pGA482-GUS)	39	33	85
	LBA4404(pSB324)	118	61	52
	LBA4404(pSB424)	109	54	50
	LBA4404(pSB124) LBA4404(pGA482) (1:1)	100	35	35
	LBA4404(pNB124) LBA(pGA482) (1:1)	100	22	22
	LBA4404(pTOK253) LBA4404(pGA482) (1:1)	110	0	0
	LBA4404(pGA482)	25	0	0
Rice	LBA4404(pTOK233)	234	199	85
	LBA4404(pSB424)	549	259	47
	LBA4404(pSB124) LBA4404(pSB4) (3:1)	49	7	14
	LBA4404(pSB124) LBA4404 (pSB4) (1:1)	82	2	2

In another study, Yan *et al*., 2003 reported that in *Agrobacterium*-mediated transformation of tobacco (*Nicotiana tabacum L.*) using *bar* gene as transgene. In single transformation, plasmid LBA4404(pDLBRBbarm) was used and the transformation efficiency was 59.2%. In co-transformation, two of plasmids LBA4404(pCMasbarm) + LBA4404(pCNPT-Ⅱ) were used with the ratio 1:1 and the transformation frequency was 20%. With the same plasmid but different ration 3: 1, LBA4404(pCMasbarm) + LBA4404(pCNPT-Ⅱ) transformation frequency increased up to 47.4% but still lower than single transformation.

4.2 Osmotic supplement of culture medium for explants

After horizontally excising the IEs, in vitro manipulation of the explant is compulsory to improve competency of plant cells to the T-DNA delivery, and to assist plant cell recovery after infection. In *Agrobacterium*-mediated transformation, osmotic treatment of enhancement largely varies depending on the species. Supplementation of co-culture medium with 68.5 g/l (200 mM) sucrose and 36 g/l (200 mM) glucose was applied extensively in rice and maize transformation (Hiei *et*

al., 1994; Zhao *et al*., 2001). However, osmotic treatment was not effective with precultured immature embryos of wheat (Uze *et al.,* 2000). Therefore, osmotic treatment did not have significant effect on T-DNA delivery in wheat. In my experiment, the IEs were precultured one week before co-cultivation and the cultured medium was supplemented with 30 g/l sucrose and it was essential to supply the nutrients that are necessary for IEs growth.

4.3 Preculture period

The preculture period is one of the considering parameters for optimizing the growth and vigour of explants that contributes for successful transformation. In rice transformation, MS or N6 was used as cultured medium (Ozawa 2009; Kumar *et al.,* 2005). The explants were usually seeds that would be triggered to become calli in the callus induction media. The time from the seeds to develop into the embryonic calli was 8 weeks and after the hard embryogenic calli appeared, they were continuously subcultured 5 days before infection with *Agrobacterium* (Kumar *et al.,* 2005). In wheat transformation, the explants were usually immature embryos and cultured on MS medium (Cheng *et al.,* 1997; Przetakiewicz *et al*., 2004). The IEs of wheat varieties were precultured 1 to 6 days prior to transformation with *Agrobacterium* (Cheng *et al.,* 1997; Haliloglu *et al.,* 2003). For IEs of oil palm, it took 5 to 7 days for the IEs to grow and attain a certain strength prior to co-cultivation. It was similar to wheat IEs, the reason IE was chosen as explant for transformation was that the time for its growth and development is faster than the leaf. For the IEs, it takes only one year to develop into the plantlet, but for the leaf as explant, it takes 5 years to regenerate into a plantlet for each single leaf was used.

The IEs in this report precultured on the N_6O for 7 days reached 3 - 4 mm in length that assisted the IEs to be familiar with the cultured environment and attain certain strength prior to be infected with *A.tumefaciens.* The browning activity was observed at some regions of the IEs as a result of the secretion of phenolic compound. This condition can be manipulated by sub-culturing the explants in the media containing active charcoal.

4.4 Co-cultivation period

The inoculation time is an important parameter that is required to be considered to increase the transformation frequency. In spring wheat varieties, Bobwhite variety was optimized by increasing the inoculation with *Agrobacterium* suspension from 30 mins to 60 mins, the co-cultivation time was from 2 to 3 days and the transformation frequency has increased from 4.4 to 4.8-19 (Hu *et al.,* 2003; Cheng *et al.,* 2003). The calli from rice were immersed in bacterial suspension for 1 min to 2 mins and the co-cultivation period were 3 days in the dark with the temperature from 25-28°C (Ozawa 2009; Kumar *et al.,*2005). The immature embryos of barley in the report of Shrawat *et al.,* 2007 was immersed in *Agrobacterium* suspension within 10-15 mins and co-cultivated for 3 days allowing the bacteria to infect the plant tissues. The OD_{600} of the barley (Shrawat *et al.,* 2007) experiment was 1 (0.5 x 10^{9} cells/ml) and the OD_{600} of rice transformation of Kumar *et al.,* 2005 was also 1, so the concentration of the bacteria is sufficient for infecting the plant tissues within 3 days. In the experiment carried out by Ozawa 2009, the OD_{600} was 0.04 and the co-cultivation is 3 days in the dark, the transformation frequency was quite high from 89% to 100%. For my experiment, the OD_{600} reached 1.8 to 1.9 for single transformation, it is very high concentration of bacteria in the suspension. The co-cultivation time in my experiment followed Ismail *et al.,* 2010, it was 30 min of co-cultivation was enough for *Agrobacterium* to infect IEs. Co-cultivation in such a short time may cause insufficient transformation and, whereas an incubation time is too long will lead to the death of explants. Therefore in my experiment, the time for co-cultivation with *Agrobacterium* was 1 day would be sufficient for transformation without causing lethal activities to the IEs. If the co-cultivation time endured more than one day, the amount of phenolic compounds (acetosyringone or hydroxyacetosyringone) would be produced excessively that lead to the death of IEs. Additionally, the density of the bacteria should be around 0.5-1. It is mostly applied (Shrawat *et al*., 2007; Kumar *et al.,* 2005 ; Ismail *et al.,* 2010)   and the parameter needs to be adjusted is the period of the co-cultivation (1 to 3 days) which is most suitable for the plant tissues to be infected with *Agrobacterium*. In many wheat varieties, the OD_{600} can reach 1 to 2 (Jones *et al*., 2005) that led to the successful transformation with the efficiency varying from 4-19% (Hu *et al*., 2003; Cheng *et al*., 2003).

4.5 Antibiotics

After incubation, the IEs in my experiment were washed with cefotaxime 250 mg/l to remove excess *A.tumefaciens* and then transferred to regeneration medium containing N_6O. In Kumar *et al.,* 2005, the rice calli were first washed with cefotaxime 250 mg/l and then transfer to the regeneration medium containing cefotaxime as well. In another report, Ozawa 2009 did the same but with the concentration of cefotaxime up to 500 mg/l, and the transformation frequency is relatively high 36 - 60% measured as numbers of transgenic rice plants regenerated. This concentration from 250 – 500 mg/l cefotaxime was appropriate for rice callus but it had a lethal effect to maize Hi II callus (Ishida *et al* .,1996). Callus formation was greatly decreased when the callus induction medium was applied with 50 to 250 mg/l, so the transformation efficiency was lower 3 times compared to that with carbenicillin (100 mg/l). Carbenicillin has been widely used in *Agrobacterium*-mediated transformation of wheat and maize (Cheng *et al.,* 1997; Zang *et al.,* 2003). Kanamycin was used in this project with the concentration 50 g/l aimed to select the bacteria that carried the desired plasmids (pCAMBIA 1302 and pCAMBIA 1302).

4.6 Temperature

The effect of temperature during co-culture of *Agrobacterium* was first reported in dicot species. An optimal temperature for T-DNA delivery was 22°C in application for tobacco leaves (Dillen *et al.,* 1997). However, in another report, highest number of transformed plants of tobacco was obtained at 25°C for co-culture period, even in 19°C can also be optimal for T-DNA delivery (Salas *et al.,* 2001). Therefore, the optimal condition for co-culture depends on specific explants and *Agrobacterium* strain. In monocots, the co-culture temperature for most plants varied from 24 to 25°C. However, in some case 28°C was still accepted for co-culture (Rasid *et al.,* 1996; Arencibia *et al.,* 1998). In case the temperature may be lower to 24°C, it has been reported the effect of lower temperature smaller than 24°C on T-DNA delivery and stable transformation. In Kondo *et al.,* 2000 report, the effect of four temperature was tested 18, 20, 22 and 24°C on T-DNA delivery with garlic calli. The highest transient expression of GUS assay was recorded at 22°C as 64% of the total calli exhibited GUS activity. The ratio of GUS stained calli reduced by 85% at 20°C and by 69% at 24°C. In comparison to the results obtained from my experiment,

single transformation and co-transformation for GUS assay were attained up to 64% and 17.39% respectively when co-culture at 25°C.

In maize, using IEs as explants, higher transformation efficiency was obtained at 20°C than at 23°C when using a standard binary vector (Frame *et al*., 2002). From the elite inbred lines PHP38 and PHN46, co-culture of IEs was carried out at 20°C followed by 28°C subculture (Gordon *et al.,* 2002). For wheat, the temperature for optimal T-DNA delivery and stable transformation was 23-25°C.

4.7 Regeneration medium

Medium component, sugar, plant growth regulators, vir induction chemicals are important factors that affect the transformation frequency. The modified N6 medium (Chu *et al*., 1995) contains 2,4-dichlorophenoxyacetic acid (2,4-D) and casamino acids was used for improving the co-culture of rice. MS and MS-based medium was widely used for regeneration of rice calli (Dong *et al.,* 1996; Lucca *et al.,* 2001). In the report of Ishida *et al*., 1996 maize IEs were regenerated using LS-based (Linsmaier and Skoog, 1965) medium and N6 based medium was unable to generate transformed plants. Zhao *et al.,* 2001 indicated that N6-based medium was added with silver nitrate would be appropriate to inoculation and regeneration of IEs of maize. In barley transformation, transformation efficiency will be enhanced if adding CaCl$_2$ to the regeneration medium (Kumlehn *et al.,* 2006).

To enhance the integration of T-DNA for wheat, salt should be reduced in the co-culture medium (Cheng *et al*., 1997). Stable transformed wheat plants regenerated from embryogenic callus were achieved in 2003 when applied the reduced salt treatment (Khanna and Daggard, 2003). Similarly, this treatment can be applied successful in maize transformation to enhance the integration efficacy, so only a half amount of salt was used in MS medium (Zhang *et al.,* 2003). In barley, the inoculation and co-culture medium were both applied with this treatment (Ke *et al.,*2002). For transient GUS expression, IEs of barley were inoculated and regenerated in one-tenth MS salt level increased up to 10 times for the GUS assay over the full-level salt.

Chemicals such as acetosyringone are advised to be used in most crops transformation protocols (Hiei *et al*., 1994; Ishida *et al*., 1996; Cheng *et al.,* 1997; Tingay *et al.,* 1997; Zhao *et al.,* 2000; Kumlehn *et al.,* 2006). When acetosyringone was absent, the transformation frequency of GUS assay was low and stable

transformants could not be regenerated in rice, onion and barley (Rashid *et al.,* 1996; Hiei *et al.,* 1997; Zeng *et al.,* 2001). However, some plants could regenerate without having addition of vir induction chemicals as compulsory. For instances, meristematic sections of sugarcane pretreated with an antinecrotic mixture (Enriquez-Obregon *et al*., 1999), and precultured IEs, embryogenic calli of wheat regeneration under desiccation conditions could be efficiently transformed (Cheng *et al.,* 2003).

In my project, N_6O medium was optimized for IEs of oil palm. Therefore, it can be used as regeneration medium so the IEs still sustained to grow for obtaining the results the following 2 weeks after incubation with *A.tumefaciens.*

CHAPTER V:

CONCLUSION

The *Agrobacterium*-mediated transformation has been established successfully on oil palm in Malaysia. The two trangenes *gus* and *gfp* were used in this experiment and the transformation efficiency of single transformation is higher than co-transformation. In co-transformation, only GUS was expressed and *gfp* genes were thought to be suppressed as the mechanism of gene silencing. This experiment gives way to further research on improving oil palm cultivar *Elaeis guineensis* Jacq. var. *Tenera* with new traits such as high yield of oil production or insect resistance genes. The preculture period was optimized in another report by Ismail *et al.,* 2010 that immature embryos (IE) require 5-7 days preculture prior to transformation. The co-cultivation period was different depending on species as reported by Ozawa 2009; Kumar *et al.,*2005 and Shrawat *et al.,* 2007. The bacterial density varies from 1 to 2 for wheat and lower in rice and barley. The co-cultivation time depending on the concentration of the bacteria, if the concentration was low, the co-cultivation period could prolong up to 3 days, whereas the concentration was high it only needed 1 day for co-cultivation as in my experiment. Many factors contribute to the transgene expression such as tissue-cultured variation, chimerism in integration site, transgene mutation and epigenetic gene silencing. Current limitation of this study was browning activity observed in transformed IEs after *Agrobacterium* infection that led to the lethality of the IEs. Consequently, the concentration of the bacteria is one of the elements should have further consideration.

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