PRELIMINARY STUDY OF *Agrobacterium*-MEDIATED TRANSFORMATION WITH POLYHYDROXYALKANOATE GENES FOR RUBBER TREE *Hevea brasiliensis* Muell. Arg. CLONE PB 350

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by

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2,4-D	2,4-dichlorophenoxyacetic acid
3HB	3-hydroxybutyrate
3-HB-CoA	3-hydroxybutyryl-CoA
3HD	3-hydroxydecanoate
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
Acetosyringone	3,5-dimethoxy-4-hydroxyacetophenone
Amp	Ampicillin
ANOVA	Analysis of variance
AtADH 5'-UTR	*Arabidopsis thaliana* alcohol dehydrogenase 5'-untranslated region
BA	Benzyl adenine
BLAST	Basic Local Alignment Search Tool
blastn	Standard nucleotide BLAST
blastx	Translated BLAST
CaMV 35-P	Cauliflower mosaic virus 35S promoter
CATAS-RRI	Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences
cDNA	Complementary DNA
CIM-A	Callus induction medium for anther

CIM-L	Callus induction medium for leaf
CIM-LC	Co-cultivation for leaf-derived callus
CIM-LD	Decontamination medium for leaf-derived callus
CIRAD	French Agricultural Research Centre for International Development
CPM	Callus proliferation medium for anther
CPM-C	Co-cultivation medium for anther-derived callus
CPM-Ca	Pre-cultivation medium for anther-derived callus
CPM-D	Decontamination medium for anther-derived callus
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9
DNA	Deoxyribonucleic acid
GAI	Gibberillic acid insensitive
GCA	General combining ability
GFP	Green fluorescent protein
GUS	β-glucuronidase
HbSOD	*Hevea brasiliensis* superoxide dismutase
HbZnCuSOD	*Hevea brasiliensis* zinc-copper superoxide dismutase
HSP-T	Heat shock protein terminator
IBA	Indolyl butyric acid
IBM	International Business Machine Corporation

LB	Lysogeny broth
LGM	Lembaga Getah Malaysia
LTC	Latex timber clone
MB	Microbouturage
MB1	MB-based SIM
MB2	MB-based co-cultivation medium
MB3	MB-based decontamination medium
Mcl	Medium-chain-length
MRB	Malaysian Rubber Board
MRT	Duncan's Multiple Range Test
MS	Murashige and Skoog
NAA	Napththalene-acetic acid
NCBI	National Centre of Biotechnological Information
NR	Natural rubber
PCR	Polymerase Chain Reaction
PGR	Plant growth regulator
PHA	Polyhydroxyalkanoate
HB	Poly(3-hydroxybutyrate)
PHBHHx	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
PLA	Poly(lactic acid)

RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic acid
RRII	Rubber Research Institute of India
RRIM	Rubber Research Institute of Malaysia
RT-PCR	Reverse transcription PCR
SALB	South American Leaf Blight
Scl	Short-chain-length
SCoT	Start Codon Targeted
SPSS	Statistical Package for Social Sciences
SR	Synthetic rubber
SRIM	Shoot and root induction medium
SSR	Simple Sequence Repeats
TAE	Tris-acetate-ethylenediaminetetraacetic acid
T-DNA	Transfer DNA
Ti	Tumour inducing
TPD	Tapping panel dryness
UV	Ultraviolet
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

KAJIAN AWAL SISTEM TRANSFORMASI BERPERANTARAKAN *Agrobakterium* DENGAN GEN POLIHIDROKSIALKANOAT BAGI POKOK GETAH *Hevea brasiliensis* Muell. Arg. KLON PB 350

ABSTRAK

Hevea brasiliensis (*H. brasiliensis*), lebih dikenali sebagai pokok getah, merupakan pengeluar utama getah asli. *H. brasiliensis* klon PB 350 merupakan salah satu klon yang disyorkan oleh Lembaga Getah Malaysia (LGM) sebagai klon lateks balak (LTC) berpenghasilan tinggi. Klon ini merupakan calon yang bagus untuk penghasilan protein heterologus kerana ia boleh menghasilkan banyak lateks dari mana protein heterologus berkenaan berkemungkinan boleh dituai. Polihidroksialkanoat (PHA) merupakan bioplastik terbiodegradasi yang dihasilkan oleh prokariot. Walaupun PHA berpotensi menggantikan plastik konvensional, kos penghasilan yang tinggi menyebabkannya tidak boleh dilaksanakan. Justeru, percubaan telah dilaksanakan untuk menghasilkan tumbuhan transgenik yang boleh menghasilkan PHA kerana sumber karbon untuk penghasilan protein heterologus dalam tumbuhan datang daripada karbon dioksida yang bebas berada di udara dan berpotensi mengurangkan kos pengeluaran. Sungguhpun kajian terdahulu telah melaporkan transformasi bagi *H. brasiliensis,* kecekapan transformasi bagi klon getah yang berbeza adalah berlainan. Justeru, tujuan kajian ini adalah untuk mengenalpasti parameter yang sesuai untuk menghasilkan *H. brasiliensis* klon PB 350 transgenik yang berupaya untuk mengekspres gen biosintetik PHA. Kajian ini bermula dengan penyaringan eksplan yang sesuai untuk transformasi. Transformasi eksplan nod yang berasal daripada anak pohon, eksplan daun daripada tumbuhan tunas cantum polibeg

dan kalus yang berasal daripada anter telah diinokulasi dengan *Agrobakterium tumefaciens* (*A. tumefaciens*) pada OD_{600} bernilai 0.6 selama 30 minit diikuti dengan ko-kultivasi selama 3 hari tidak berjaya menghasilkan *H. brasiliensis* trangenik. Transformasi kalus anter yang lebih mudah untuk pensterilan permukaan, boleh diperoleh dalam jumlah yang banyak dan boleh menjana progeni yang sama dengan klon induk secara genetik telah dilaksanakan. Walaupun 5.0 mg/L higromisin B bersendirian adalah mencukupi untuk menghalang tumbesaran kalus dari anter PB 350 yang taktertransformasi, 500 mg/L cefotaxime berupaya menggalakkan tumbesaran kalus yang taktertransformasi apabila ia turut dicampur ke dalam media mempunyai higromisin untuk menyingkir *Agrobakterium* . Suatu reka bentuk ujikaji faktorial yang mengandungi 100 kombinasi parameter yang berbeza iaitu ketumpatan bakteria (OD_{600} = 0.2, 0.4, 0.6, 0.8), masa inokulasi (1s, 15s, 30s, 45s, 60s) dan tempoh masa ko-kultivasi (1 hari, 2 hari, 3 hari, 4 hari, 5 hari) telah dilaksanakan. Keputusan menunjukkan bahawa inokulasi kalus dari anter dengan *A. tumefaciens* pada OD_{600} pada 0.2 untuk 1 saat dan ko-kultivasi selama 1 hari memberikan hasil terbaik dari segi memperoleh kadar kontaminasi *Agrobakterium* yang paling rendah (0%), penghasilan kalus-kalus tahan antibiotik setinggi 33% dan kecekapan transformasi paling tinggi (22.2 ± 9.6%). Transformasi dan pengekspresan gen yang dimasukkan juga telah disahkan melalui PCR dan transkripsi berbalik PCR. Transformasi stabil dan bilangan salinan gen tidak dapat disahkan kerana analisis blot Southern tidak dilaksanakan.

PRELIMINARY STUDY OF *Agrobacterium*-MEDIATED TRANSFORMATION WITH POLYHYDROXYALKANOATE GENES FOR RUBBER TREE *Hevea brasiliensis* MUELL. ARG. CLONE PB 350

ABSTRACT

Hevea brasiliensis (*H. brasiliensis*), better known as rubber tree, is the main producer of natural rubber. *H. brasiliensis* clone PB 350 is one of the clones recommended by the Malaysian Rubber Board (MRB) as a high yielding latex timber clone (LTC). This clone is a good candidate for heterologous protein production because it can produce high amount of latex from where the heterologous protein could potentially be harvested from. Polyhydroxyalkanoate (PHA) is a biodegradable bioplastic produced by prokaryotes. While PHA has the potential to replace conventional plastics, the high cost of production makes it not feasible. Thus, attempts had been made to generate PHA-producing transgenic plants because carbon sources for heterologous protein production in plant comes from freely available carbon dioxide in the air and can potentially lower the production cost. Eventhough previous studies have reported on the transformation of *H. brasiliensis*, the transformation efficiency of different rubber clones differ. Therefore, the aim of this study was to determine suitable parameters to produce transgenic *H. brasiliensis* clone PB 350 capable of expressing the PHA biosynthetic genes. This study began with the screening for suitable explants for transformation. Transformation of seedling-derived nodal explants, leaf explants from bud-grafted polybag plants and anther-derived calli that were inoculated with *Agrobacterium tumefaciens* (*A. tumefaciens*) at OD_{600} of 0.6 for

30 minutes followed by 3 days of cocultivation was unsuccessful in producing transgenic *H. brasiliensis.* Transformation of anther-derived calli that were easier to surface sterilize, can be obtained at a higher number and could generate progenies which are genetically identical to the maternal clone was then conducted. Although hygromycin B at 5.0 mg/L alone was found to be sufficient to prevent the growth of untransformed anther-derived calli of PB 350, 500 mg/L cefotaxime was able to promote the growth of the untransformed calli in the presence of hygromycin when it was also added into the media to eliminate *Agrobacterium.* A factorial experimental design consisting of 100 different combinations of parameters consisting of bacterial density (OD_{600} = 0.2, 0.4, 0.6, 0.8), inoculation time (1s, 15s, 30s, 45s, 60s) and co-cultivation period (1 day, 2 days, 3 days, 4 days, 5 days) was performed. The results showed that inoculation of anther-derived calli with *A. tumefaciens* at OD_{600} of 0.2 for 1 s and co-cultivation for 1 day resulted in the lowest *Agrobacterium* contamination rate (0%), antibiotic-resistant calli generation up to 33.3% as well as the highest transformation efficiency (22.2 ± 9.6%). Transformation and expression of polyhydroxyalkanoate biosynthetic genes were also confirmed *via* PCR and reverse transcription PCR. Stable transformation and copy number of genes could not be confirmed because Southern blot analysis was not performed.

CHAPTER 1

INTRODUCTION

1.1 Background

Hevea brasiliensis (*H. brasiliensis*) or commonly known as the rubber tree belongs to the family of Euphorbiaceae. It has an abundance of highly specialized laticifer cells in the bark, which are formed in rings interspersed with sieve tubes. Upon tapping of the bark, latex, a specialized cytoplasm, could be obtained. Within the latex, natural rubber (NR) particles, lutoids and Frey-Wyssling particles could be found (Bottier, 2020). While there are other plant species which could similarly produce NR-containing latex such as guayule and the Russian dandelion, *H. brasiliensis* had become the major producer of NR due to its capability to produce a high yield of high molecular weight NR (Hayashi, 2009).

Plant transformation system for the production of heterologous proteins had gained more popularity in the recent years because it promises an inexpensive cost as well as the ease of scaling up (Twyman et al., 2003) and owing to the capability of rubber tree in producing large volume of latex, it has become an interesting candidate as a host for heterologous protein production in plants. This is because while most plants require the disruption of either portions or the entirety of the plants upon harvesting of the heterologous proteins, harvesting of heterologous proteins from a rubber tree, on the other hand, is a non-destructive process whereby the proteins could be recovered from the tapping of the tree throughout the year. This had been demonstrated by Arokiaraj et al. (1998) whereby transformation of *β-glucuronidase* (*gus*) reporter gene into *H. brasiliensis* clone GL 1 had shown expression in the latex

by making the latex turn blue when tested with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (*X*-gluc), a GUS substrate.

As such, several studies have been conducted on making use of the extensive laticiferous system of this tree for the production of other desired products. In Malaysia specifically, the Malaysian Rubber Board (MRB) had worked on several studies involving the use of rubber tree for heterologous protein production. Yeang et al. (2001) showed the expression of a functional recombinant antibody fragment in the latex of transgenic *H. brasiliensis*. A year later, Arokiaraj et al. (2002) successfully transformed the rubber tree to produce human serum albumin. Later, they had also performed transformation on calli derived from the anther of *H. brasiliensis* clone GL 1 for heterologous production of human protamine 1 and human atrial natriuretic factor (Sunderasan et al., 2010, 2012).

However, only a few studies had reported on transformation of *H. brasiliensis* for the purpose of heterologous protein production. Most researchers working on *H. brasiliensis* do so with the aim of improving the rubber tree as a NR producer, such as *via* improving the resistance of this crop tree towards stress such as tapping panel dryness (TPD, also known as brown bast syndrome), a condition generally found on rubber trees which suffered from oxidative stress due to excessive tapping as well as water deficit. The Rubber Research Institute of India (RRII), for example, had introduced *H. brasiliensis superoxide dismutase* (*HbSOD*) gene into *H. brasiliensis* clone RRII 105 for the very purpose of alleviating the symptoms of TPD (Jayashree et al., 2003). Another group of researchers from the French Agricultural Research Centre for International Development (CIRAD) had performed transformation on *H. brasiliensis* clone PB 260 using *H. brasiliensis zinc-copper superoxide dismutase*

(*HbZnCuSOD*) gene to improve the tolerance of this crop plant towards water deficit (Leclercq et al., 2012).

Ideally, an effective heterologous-protein-producing rubber tree would be one which is high-yielding. Therefore, it is necessary to perform transformation on elite clones of *H. brasiliensis*. However, in most of the successful transformation works for heterologous protein production in rubber tree, as had been performed by the MRB, the clone used was limited to GL 1, an old clone which has low latex yield (Gooding, 1952) and therefore it was expected to result in low yield of heterologous proteins after transformation. Up to now, there are no reports on the efficient transformation protocol for high-yielding *H. brasiliensis*. This is because elite clones are more recalcitrant to somatic embryogenesis (Enjalric and Carron, 1982), a step necessary for the production of non-chimeric transgenic plant.

Therefore, in this study, the focus was to establish an effective protocol for the transformation of *H. brasiliensis* clone PB 350. This clone is an MRB-recommended high yielding Malaysian latex timber clone (LTC). In this study, several explants namely anther-derived calli, leaves and nodal segments were tested for their suitability for transformation. Genes for the biosynthesis of polyhydroxyalkanoate (PHA), a kind of biodegradable bioplastics, were subsequently transformed into this clone and successful transformation was confirmed *via* visual and molecular techniques. PHA biosynthetic genes have been chosen for the transformation because *H. brasiliensis* as a latex-producing plant has a naturally high flux of acetyl-coenzyme A, which also happens to be the first substrate for PHA biosynthesis (Dalton et al., 2013). It will be interesting to study the effects of these genes on the growth of *H. brasiliensis*. It was hypothesized that generation of transgenic *H. brasiliensis* PB 350 would be possible

despite it being a high-yielding LTC as long as suitable transformation parameters could be established.

1.2 Objectives of this study

1. To screen for suitable explant for *Agrobacterium*-mediated transformation of *Hevea brasiliensis* clone PB 350;
2. To perform preliminary optimization of *Agrobacterium*-mediated transformation of anther-derived calli of *Hevea brasiliensis* clone PB 350 with polyhydroxyalkanoate biosynthetic genes, *phaC*, *phaA* and *phaB*;
3. To confirm the transient polyhydroxyalkanoate gene transformation into anther-derived calli of *Hevea brasiliensis* clone PB 350 *via* green fluorescent protein screening and polymerase chain reaction.

CHAPTER 2

LITERATURE REVIEW

2.1 *Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.

2.1.1 Economic importance of *H. brasiliensis*

H. brasiliensis is the main source of natural rubber (NR). Other plant species other than the rubber tree which are known to produce large quantity of NR are guayule (*Parthenium argentatum* Gray) and the Russian dandelion (*Taraxacum koksaghyz*) (Hayashi, 2009). These plant species however could not replace *H. brasiliensis* as they suffer from several setbacks. Guayule, for example, produces rubber which is less stable to oxygen and heat when compared to those produced by the rubber tree (Schloman, 2005). On the other hand, the Russian dandelion was found to produce rubber which contain possibly more associated proteins than those produced by rubber tree. This indicates that people are more likely to experience allergic reaction when in contact with rubber produced by the Russian dandelion than to rubber from the rubber tree (Cornish et al., 2005). Furthermore, unlike *H. brasiliensis* which could be tapped to obtain the NR contained in the latex, NR from guayule and the Russian dandelion can only be obtained through destructive harvesting of the plant materials such as the branches of guayule and the roots of the Russian dandelion (Van Beilen and Poirier, 2007).

NR is used for the production of various products such as tires, medical devices, surgical gloves and other consumer products due to properties such as elasticity, resilience, water resistance, durability and toughness (Lim, 2012). Of these, the transportation industry alone accounts for over 50% of NR utilisation (Hayashi, 2009).

While there is competition between NR and synthetic rubber (SR), the demand for NR has continued to increase over the years (The Rubber Economist, 2020). This is because while SR could be produced at a lower cost than NR, NR's superior tear strength and heat-resistance make it better-suited for high-performance tires used on trucks, busses, aircrafts, and racing cars (Hayashi, 2009). Until 2012, Malaysia was among the 3 largest producers of NR along with Thailand and Indonesia. However, as of 2019, Malaysia had dropped to the seventh position in NR production with only 639 830 tonnes of NR produced in that particular year (FAO, 2021).

Besides NR, *H. brasiliensis* could also be used to produce rubberwood. This is especially true for latex timber clones (LTCs) which are bred for their latex and timber. Originally used as fuel for drying and smoking rubber as well as being a source of charcoal for local cooking, rubberwood had over time being used for manufacturing furniture, wooden toys and boxes as well. This is due to its dense grain, minimal shrinkage, attractive colours and ease at accepting different finishes. It is also thought to be an environmentally friendly wood since the wood is obtained from trees at the end of their latex-producing cycle. In 2017, Malaysia ranked the 9th largest furniture exporter in the world with 80% of these made from rubberwood (Ratnasingam et al., 2018). In some cases, *H. brasiliensis* is grown for its timber with latex as the co-product (Van Beilen and Poirier, 2007; Lim, 2012).

2.1.2 *H. brasiliensis* clone PB 350

New *H. brasiliensis* clones are constantly being generated in order to increase latex yield and in recent years, timber yield. In Malaysia, the rubber breeding programme begun as early as 1928 at the Rubber Research Institute of Malaysia

(RRIM). It was found that when crosses were made between related parents, inbreeding depression and unpredicted interaction occurred (Tan et al., 1975). Furthermore, general combining ability (GCA) estimates for clonal yields can be used as a criterion for parents selection (Tan, 1977), whereby GCA refers to the average performance of a genotype in a series of cross combination and is directly related to the breeding value of a parent associated with additive genetic effects (Rukundo et al., 2017).

PB 350, is a cross between RRIM 600 and PB 235. The parents, RRIM 600 and PB 235, are both relatively high latex yielders with moderate to large girth. They are moderately affected by wind damage and TPD. While PB 235 is highly susceptible to *Oidium*, RRIM 600 is tolerant (Priyadarshan et al., 2009). Furthermore, they are not closely related with the parents of RRIM 600 being Tjir 1 and PB 86 while the parents of PB 235 are PB 5/51 and PB S/78 (Sant'Anna et al., 2021). PB 350 was a class I clone recommended for large-scale planting at any regions (Wei and Shamsul Bahri, 2014) under the RRIM Planting recommendations 1995, and remained a class I clone up until the RRIM Planting recommendation 2013. Class I clones are clones with 5 years data showing an average minimum yield of 1 500 kg/ha/yr, obtained through the large scale clone trial and possess desirable secondary traits such as thick bark as well as resistance to wind damage, TPD and leaf diseases. PB 350 in particular has a 5-year average yield of 1 525 kg/ha/yr and has good resistance against wind damage as well as *Oidium* and *Colletotrichum*. The leaves of PB 350 are rounded, light green in colour, dull and smooth. The apex of the leaves is cuspidate with an obtuse base. They have wavy sides. The seeds are oblong in shape, medium-sized, glossy and dark brown in colour (MRB, 2013).

2.2 Plant tissue culture

2.2.1 General overview of plant tissue culture

Plant tissue culture is a method for *in vitro* growing of isolated parts of different plant species whether they are cells, tissues, or organs, on artificial media under aseptic and defined physical as well as chemical conditions based on the theory of plant cell totipotency (Thorpe, 2012). The theory of totipotency refers to the capability of cells to regenerate and form an intact plant (Su et al., 2020). The first ever plant tissue culture was by Gottlieb Haberlandt in 1902, who had attempted to culture hair cells of plant leaf mesophyll tissue. His attempt, however, was unsuccessful because of the lack of growth regulators which prevented the cells from dividing. Nevertheless, Haberlandt is recognized as the father of plant tissue culture on the basis of his pioneer experiment (Yancheva and Kondakova, 2018). Plant tissue culture can be categorized under cultures of organized structures such as meristems, shoots or shoot tips, nodes, isolated roots and embryos as well as cultures of unorganized tissues which include callus cultures, suspension cultures, protoplast cultures and anther cultures (George et al., 2008b).

Some of the basics of plant tissue culture include selection of explant, surface sterilization, inoculation in suitable culture medium under controlled conditions as well as acclimatization (Loyola-Vargas and Ochoa-Alejo, 2012). Explants are small organs or pieces of tissue used to start a tissue culture. The explants used depend on factors such as the kind and purpose of culture as well as the plant species to be used. As such, there can be many different kinds of explants and the correct choice of explant would affect the success of the culture (George et al., 2008b). In plant tissue culture, it is essential for the culture to be contaminant-free. Contaminants may influence the

culture by releasing metabolites which affect the plant directly or by altering the composition and pH of the culture medium. In addition, the contaminants may overrun the culture due to the richness of the medium. Therefore, surface sterilization of the explant should be sufficiently performed (Cassells, 2012).

For healthy growth, plants need to take up various nutrients from the soil. These include macronutrients needed in large amounts such as nitrogen, potassium, calcium, phosphorus, magnesium and sulphur as well as micronutrients needed in smaller quantities such as iron, nickel, chlorine, manganese, zinc, boron, copper and molybdenum. In plant tissue culture, however, these nutrients are supplied *in vitro*, on artificial media (George et al., 2008a). The most commonly used medium is the Murashige and Skoog (MS) medium which was initially developed for tobacco cultures (Murashige and Skoog, 1962). This medium formulation can then be modified to suit the needs of different plant species which have different characteristic elementary composition. A major problem with medium modification is precipitation, which typically occur following autoclaving due to it being an endothermic process. Other than the inorganic components provided by the macro and micronutrients, carbon in the form of sugar, plant growth regulators (PGRs) and other additives such as vitamins or coconut water may also be added into the culture medium (George et al., 2008a). For semi-solid media, gelling agents such as agar and gellan gum are also added into the media (George et al., 2008b).

2.2.2 Tissue culture of *H. brasiliensis*

The most used method for the propagation of rubber tree is grafting. However, even under the best management conditions, uniform growth and yield could not be

achieved. This may be attributed to the heterogeneity of the rootstocks used and the rootstock-scion interaction as shown by Cardinal et al. (2007) whereby different combinations of rootstocks and scions could lead to varying rubber yield. Therefore, the propagation of elite clones with their own root systems is highly desirable as this could reduce intra-clonal variation caused by stock-scion interaction and this could be achieved *via* tissue culture. *In vitro* culture works in *Hevea* mostly encompassed micropropagation *via* nodal cultures, shoot tip cultures, somatic embryogenesis and genetic transformation (Nayanakantha and Seneviratne, 2007).

2.2.2(a) Micropropagation of *Hevea via* nodal and shoot tip cultures

In 1976, Paranjothy and Ghandimathi had for the first time attempted seedling-derived shoot tip culture and managed to obtain rooted shoots in liquid MS medium which unfortunately failed to grow on semi-solid medium (Nayanakantha and Seneviratne, 2007). Later, shoots derived from 1 – 3 year old greenhouse grown seedlings were successfully used to develop rooted plantlets with the aid of benzyl adenine (BA), indolyl butyric acid (IBA), naphthalene-acetic acid (NAA) and activated charcoal (Enjalric and Carron, 1982). Thereafter, different researchers had successfully obtained rooted plantlets from seedling-derived shoots. For example, Gunatilleke and Samaranayake (1988) reported success in generating rooted plantlets from shoots derived from seedlings of *in vitro* germinated seeds of *Hevea* clone PB 86 while Antwi-Wiredu et al. (2018) reported the induction of shoot (up to 94 %) and root (percentage not mentioned) from shoots and nodal explants of their local clone.

Explants derived from elite *Hevea* clones, however, are highly recalcitrant and as such, reports on successful micropropagation using clonal materials are very limited.

The major issues faced with this approach were the failure to produce tap root system adequate for tree stability (Enjalric and Carron, 1982) as well as *in vitro* proliferation which was severely lacklustre (Seneviratne, 1991). Perrin et al. (1997) demonstrated that using clonal material such as nodal explants from RRIM 600, PB 260 and IRCA 18, the percentage of explants producing shoots were 38.5 % for RRIM 600 and 0.6 % for IRCA 18 while rooting was seen only in clone IRCA 18 at 2.3 %. However, the type of root produced whether fibrous root or tap root was not mentioned. Furthermore, from their study, micropropagation of PB 260 produced neither shoots nor roots. Using RRIM 2020 regenerated from somatic embryos, Nor Mayati and Jamnah (2014) reported that there were poor growth of induced shoots and no root induction. Furthermore, following the second subculture, all the cultures were contaminated and therefore discarded. Similarly, micropropagation using clone RRIM 2025 had only been reported to produce shoots without any root formation (Moradpour et al., 2016).

2.2.2(b) Somatic embryogenesis of *Hevea*

In plants, somatic cells can generate embryo-like structures without going through fertilization. These structures are called somatic embryos and since fertilization event is not involved in their production, they are essentially clones which are genetically identical to the parent plant (Deo et al., 2010). Plant regeneration *via* somatic embryogenesis is important not only for the purpose of micropropagation but also as a platform for the generation of transgenic plants. This is because plants propagated from shoot or nodal cultures would form chimeras with different genotypes within the tissues (Lardet et al., 2011). Somatic embryos could be obtained from either direct somatic embryogenesis whereby embryogenic cells develop directly from the

explant, or indirect somatic embryogenesis whereby the explant first forms dedifferentiated clumps of calli before forming embryogenic cells (Merkle et al., 1990; Mignon and Werbrouck, 2018). In *Hevea*, however, instances of somatic embryogenesis were mostly if not all indirect. The indirect somatic embryogenesis process proceeds through four successive phases which are callogenesis, differentiation, multiplication of embryos, and development of embryos into plantlets (Carron et al., 1989).

Somatic embryogenesis of *Hevea* could be initiated from various kind of explants. Anther is one of the most reported sources of somatic embryo. Anther-derived calli could be initiated from either the wall of the anther or from the pollen grain inside the anther as was described by the first ever *Hevea* anther culture performed by Satchuthananthavale and Irugalbandara (1972) using clone RRIC 52 in an attempt to generate haploid plants. However, they did not report shoot or root induction from the calli culture. Satchuthananthavale (1973) reported that the calli initiated from anther culture from either the anther wall or pollen grain could be a mixture of diploid and haploid cells, rendering their attempt at obtaining haploid plants impossible. In 1974, a Malaysian researcher, Paranjothy reported successful differentiation of embryos from anther wall derived calli for the first time (reviewed by Nayanakantha and Seneviratne, 2007). Years later, Wang et al. (1980) reported success in obtaining normal plantlets of *H. brasiliensis* clone Hai Ken 2, Hai Ken 1 and SCATC 88-13 *via* somatic embryogenesis of anther-derived calli. Cytological studies suggested that the somatic embryogenesis plantlets were originated from somatic cells, presumably the anther walls. Besides Sri Lankan, Malaysian and Chinese researchers, Thailand researchers (Te-chato and Aengyong, 1988) had also reported success on using uninucleate or late uninucleate stage anther for anther

cultures of clones RRIM 600, PR 255 and GT 1 to obtain diploid plantlets despite experiencing slow shoot growth. Later, India also reported success in somatic embryogenesis and plant regeneration from immature anther culture of their own rubber clone, RRII 105 (Jayasree et al., 1999). Even now, somatic embryogenesis from anther cultures are still being practiced. For example, there were reports of somatic embryogenesis and plantlet development from anther of rubber clone RRII 105 (Jayashree et al., 2003), PR 107, RRIM 600, Reyan 8-79, Haiken 2 (Tan et al., 2011), unknown clones (Sirisom and Te-chato, 2013), 2-nr, 6-nr, 1-em, 1-tF (Srichuay et al., 2014), Reyan 7-33-97 (Tan et al., 2011; Zhao et al., 2015) and RRII 414 (Jayasree and Rekha, 2019).

Another explant regularly used for initiating somatic embryogenesis in *H. brasiliensis* is the immature inner integument. Most of the research done on using immature inner integument of *H. brasiliensis* for somatic embryogenesis were performed by researchers from the French Agricultural Research Centre for International Development (CIRAD) using primarily clone PB 260 (Carron et al., 1998; Lardet et al., 1999; Blanc et al., 2002; Lardet et al., 2007) and occasionally other clones such as PB 235, PR 107, RRIM 600 and GT1 (Montoro et al., 1993). CIRAD's collaborator from Indonesia had also performed somatic embryogenesis from immature inner integument using the exact same clone, PB260 (Bintarti, 2015). Collectively, they had studied the effects of basal media composition and PGRs on somatic embryogenesis and plantlet regeneration from immature inner integument as well as establishing cryopreservation process for long-term storage of embryogenic calli which retain their ability to regenerate into plantlets. Another French group had also used clone PB 260 to study the effect of fruit shelf-life on callogenesis and somatic embryogenesis from inner integuments (Modeste et al., 2012). Chinese researchers had

also conducted somatic embryogenesis and plantlet regeneration from immature inner integument such as shown by Li et al. (2010) using clone Reyan 88-13 and Sun et al. (2012b) using clones Reyan 88-13 and Reyan 7-33-97. Beside these, Thai researcher had also reported success in using immature inner integument of clone RRIM 600 for the initiation of somatic embryo and subsequently plantlet regeneration as well as confirming that the regenerated plantlets were true-to-type to the mother plant *via* three Polymerase Chain Reaction (PCR)-based techniques, Random Amplified Polymorphic DNA (RAPD), Start Codon Targeted (SCoT) and Simple Sequence Repeats (SSRs) (Tisarum et al., 2020).

Other less commonly used explants for somatic embryogenesis in *H. brasiliensis* include leaves, roots and ovules. While there were many works performed on using leaves for somatic embryogenesis in other plant species such as cassava (Susanti et al., 2017; Marius et al., 2018), jatropha (Laguna et al., 2018; El-Sayed et al., 2020; Gudeta et al., 2020), and papaya (Cipriano et al., 2018; Malik et al., 2019), there were limited reports on the use of this explant in *H. brasiliensis* by the Rubber Research Institute of India (RRII) using clone RRII 105. Despite the limited reports on the use of this explant, one thing that was certain was that the age of the leaves played a significant role in the success using this explant (Kala et al., 2009; 2015). The stages of leaves were summarised by Hallé and Martin (1968) as having 4 stages: A) bud break, B) growth, C) leaf maturation and D) dormancy. These stages are as shown in Figure 2.1. While the Rubber Research Institute, Chinese Academy of Tropical Agricultural Sciences (CATAS-RRI) did attempt to generate somatic embryos from leaf explants, the leaf explants they had used were obtained from seedlings and were therefore not clonal (Sun et al., 2012a).

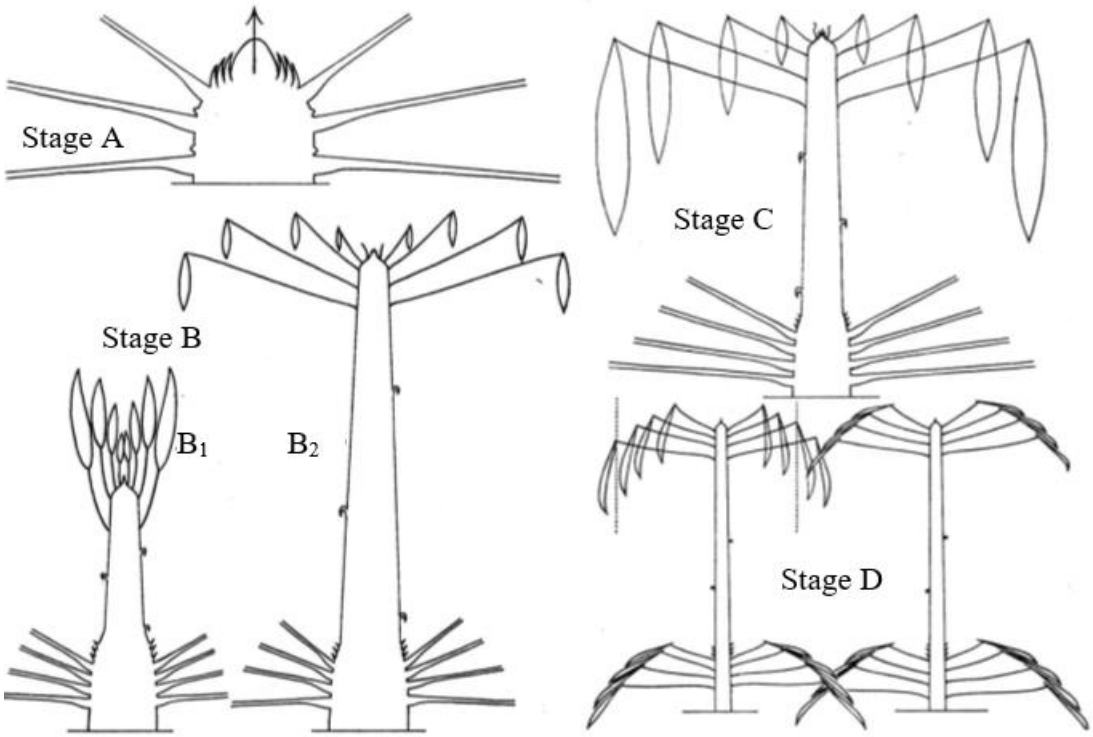

Figure 2.1 Stages of leaves of *Hevea brasiliensis*. Image obtained from Hallé and Martin (1968).

Similar to leaves, reports on the use of roots and ovules for somatic embryogenesis were very limited. The use of roots for somatic embryogenesis was demonstrated by Sushamakumari et al. (2014) who had obtained the root explants from somatic embryos of clone RRII 105 originally initiated from anthers. For ovules, their use for somatic embryogenesis and regeneration into plantlets were demonstrated by Chen et al. (1988) and Jayashree et al. (2011) in the attempt to produce haploid plants.

Despite all the studies done, the frequency of plant regeneration was still considerably low, rendering it not viable for commercial scale application. Somatic embryogenesis in *H. brasiliensis* is highly genotype-dependent and the low rate of plantlets conversion from the embryos necessitates the individual optimization of the culture conditions for each and every clone of *H. brasiliensis* (Carron et al., 1995).

2.3 Plant genetic transformation

2.3.1 Overview of plant genetic transformation

Genetic transformation entails the introduction and subsequent expression of foreign genes in a host organism. The transformation could be transient or stable which means it could be either temporary and uninheritable or it could be incorporated into the genome and becomes inheritable, respectively (Handler, 2008). In plants, genetic transformation allows for the introduction of agronomically useful genes into crops and provides the tools for producing novel and genetically diverse plant materials (Keshavareddy et al., 2018). Furthermore, advancements in plant genetic engineering have made it possible to transfer not only plant genes into crops, but also genes from non-plant organisms, making transgenic plants a viable alternative for the production of recombinant proteins and vaccines. However, there remain some crop plants in

which an efficient transformation method is lacking, thus being the bottleneck towards the application of plant transformation in these plants (Barampuram and Zhang, 2011).

2.3.2 *Agrobacterium*-mediated transformation

There exist various methods for plant transformation which include electroporation into protoplasts, microinjection, *in planta* transformation, particle bombardment, *Agrobacterium*-mediated transformation and more recently, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) system (Gosal and Wani, 2018). *Agrobacterium*-mediated transformation takes advantage of the natural capability of *Agrobacterium tumefaciens* (*A. tumefaciens*) to transfer a segment of its genetic material located on the tumour inducing (Ti) plasmid, known as the transfer DNA (T-DNA), into plant nucleus and incorporate the DNA stably within the genome (Chilton et al., 1977). While this transformation method has become widely used for transgenic plants production, various challenges still remain especially for genotype-independent transformation of economically important crop plants (De La Riva et al., 1998).

Previously found to be essential for inducing tumours in plants, a large supercoiled circular plasmid (more than 200 kb) was hypothesised to carry the genetic information necessary for crown gall induction. These include the T-DNA region which is stably integrated into the host following successful transfer and the *vir*-genes which induce the virulence (Agrawal and Rami, 2022). Van Larebeke et al. (1974) showed that curing of these plasmids, called tumour-inducing (Ti) plasmid, resulted in 100% loss in the tumour-inducing capability of the *Agrobacterium*. Hoekema et al. (1983), however, later showed that while both the T-DNA region and the *vir*-genes are

necessary to induce tumorigenesis, they do not necessarily have to be in the same plasmid. This opened the avenue to easily manipulate a much smaller plasmid containing only the T-DNA region while the helper plasmid containing the *vir*-genes can be inserted into the bacterium beforehand. The two smaller plasmid which are self-sufficient yet interdependent for transformation constitute the binary system. Easily manipulated, this system had been shown to be useable for transfer of multiple genes simultaneously up to 150 kb (Hamilton et al., 1996; 1997).

While the plasmids used for *Agrobacterium*-mediated transformation lost their native tumour-inducing ability, the mechanism by which the DNA fragments from *Agrobacterium* get transferred into host cells remained the same. The process includes recognition and response of *Agrobacterium* to host, production of T-DNA and transport of the transferred substrates from the bacterium to the host cell, travelling of T-DNA in host cell, and integration of T-DNA into host genome (Guo et al., 2019). The process starts when host cells produce specific signals such as phenolic compounds. Wounding promotes T-DNA transfer as cell repair in plants induces the production of phenolic compounds. In response to these signals, VirA/VirG activates a cascade of other *vir* protein machinery genes (Nester, 2015). Subsequently, VirD1 and VirD2 proteins proceed to nick the left and right borders of the bottom strand of the T-DNA, resulting in a single stranded T-DNA molecule known as the T-strand. This T-strand remains bonded covalently to the VirD2 upon cleavage to form VirD2-T-strand nucleoprotein complex (Scheiffele et al., 1995).

While these happen, the bacterium adheres to the host cell surface *via* several binding mechanism, one of which is mediated by T-pillus assembled by VirB2 and VirB5. VirD4 and VirB protein complex then form channels through which the VirD2-T-strand complex is then exported along with several vir proteins, one of which is

VirE2, into the host cell cytoplasm (Christie et al., 2014). VirE2, a single stranded DNA-binding protein whose function is presumably to prevent the degradation of the T-DNA upon entry into the host cell cytoplasm then proceed to coat the VirD2-T-strand complex forming a nucleoprotein complex termed T-complex (Lacroix and Citovsky, 2013). VirD2 and VirE2 may play important role in targeting the T-complex to host nucleus due to their nuclear localization signal (Yang et al., 2017). The T-strand is later integrated into the host genome at random positions through illegitimate recombination (Barampuram and Zhang, 2011). The whole process is summarized in Figure 2.2. In addition to VirA – VirE, there exist at least four other virulent proteins, VirF, VirG, VirH1 and VirH2. These proteins are thought to assist in the transfer of T-DNA into a plant host by either detoxification of plant chemicals which interfere with bacterial growth or by defining bacterial strain host range specialization. However, the actual functions and mechanisms of these proteins are not well understood (Agrawal and Rami, 2022).

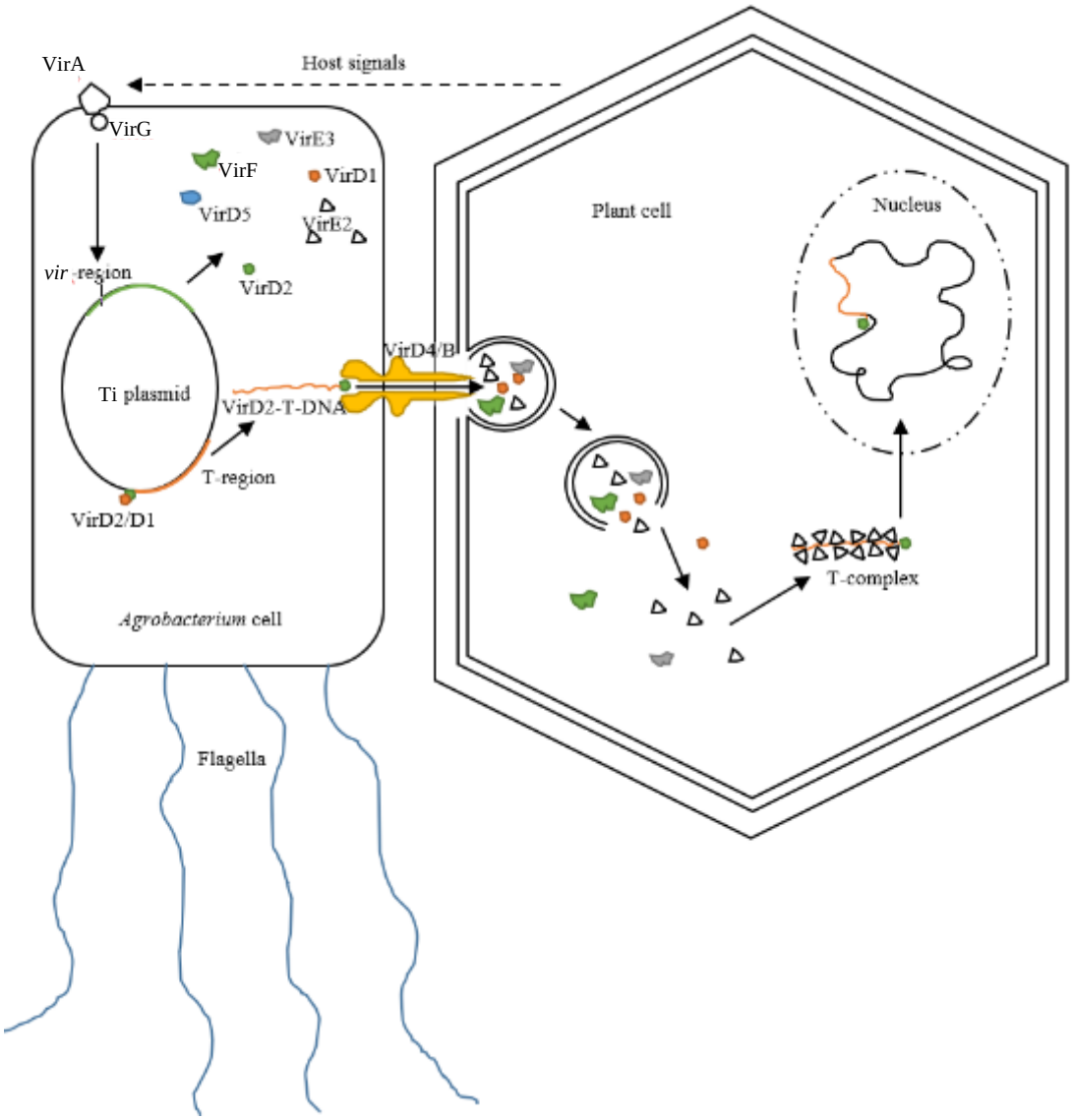

Figure 2.2 Simplified diagram of the mechanism of *Agrobacterium*-mediated T-DNA transfer. Image adapted from Guo et al. (2019).

2.3.2(a) Factors affecting *Agrobacterium*-mediated transformation

Many factors affect the efficiency of *Agrobacterium*-mediated transformation. The strain and concentration of *Agrobacterium* used are among these factors. Efficient *Agrobacterium*-mediated transformation of not only different plant species but also different varieties or clones within the same plant species requires the use of different strain and concentration of *Agrobacterium.* Based on the study by Montoro et al. (2000), *Agrobacterium*-mediated transformation of *H. brasiliensis* clone PB 260 using 5 strains of *Agrobacterium* namely C58pMP90, C58GV2260, AGL1, LBA4404 and EHA105 had shown that regardless of the plasmid used (either pGIN or pCAMBIA2301), EHA105 showed higher transformation efficiency compared to the rest of the strains. Nyaboga et al. (2015), on the other hand, reported that for cassava cultivar TME14, the strain (either LBA4404 or EHA105) showed no significant difference but the best concentration for efficient transformation was OD_{600} at 0.25.

Inoculation time is the time whereby the explant would be immersed in the *Agrobacterium* suspension before they are co-cultivated on semi-solid medium. Among the longest inoculation time taken for transformation was 3 hours as reported by Amoah et al. (2001) for wheat. On the lower end, a short inoculation time of mere 1 s was reported for the transformation of rubber tree calli (Montoro et al., 2003).

Another factor crucial for the successful generation of transgenic plant is the co-cultivation period. The co-cultivation period should be optimized to allow for the interaction between *Agrobacterium* and the explant, thus potentially increasing the chance for genetic material transfer into the explant while controlling *Agrobacterium* overgrowth (Zhang and Finer, 2016). Guo et al. (2012) reported the use of 1 day co-cultivation for tomato cultivar Micro-Tom transformation while an extremely long co-

cultivation period of up to 15 days was reported by Zhang and Finer (2016) for the generation of transgenic sunflower.

Following co-cultivation, *Agrobacterium* should be eliminated from the explant to prevent overgrowth of the bacterium which may affect explant viability and growth. The use of suitable antibiotic could promote higher transformation efficiency. β-Lactam antibiotics such as carbenicillin, cefotaxime, meropenem, moxalactam and timentin are commonly used since these kind of antibiotics inhibit prokaryotic cell wall synthesis while showing little detrimental effect on eukaryotic plant cells (Ogawa and Mii, 2007).

The choice of antibiotic used will affect not only the elimination of *Agrobacterium*, but also potentially explant growth. This is evident from the work done by Nauerby et al. (1997) whereby they have studied the effect of three antibiotics, namely timentin (150 mg/L), cefotaxime (500 mg/L) and carbenicillin (100 mg/L), at concentrations suitable for the elimination of *Agrobacterium*, on the regeneration potential of *Nicotiana tabacum* Petit Havana SRI leaf disc and cotyledon explants. In their study, shoot regeneration from leaf disc was found to be positively affected by timentin, negatively affected by carbenicillin and not significantly affected by cefotaxime although rooting of shoots regenerated from leaf disc was inhibited by cefotaxime. Cotyledon explant regeneration, on the other hand, was not affected by timentin but negatively affected by carbenicillin and cefotaxime.

After elimination of *Agrobacterium*, explants must undergo selection to identify putatively transformed explants. The most commonly used method of selection is the negative selection using antibiotics and herbicides to prevent growth of untransformed explants. Commonly used antibiotics include, kanamycin,

paromomycin, hygromycin and phosphinothricin while glyphosate is the most commonly used herbicide (McCormick et al., 1986; Aoki et al., 2002; Hu et al., 2003; Luo et al., 2004; Joyce et al., 2010).

In addition, explant age is important because it determines the amenability of explants towards transformation. Generally, the younger the explant, the more responsive it is to transformation. *Agrobacterium*-mediated transformation of apple (*Malus* × *domestica* Borkh), for instance, was found to be most efficient when 25-day-old shoots were used and decreases for shoots older than 25 days old (De Bondt et al., 1994). In addition, Mannan et al. (2009) showed that *Agrobacterium*-mediated transformation of *Artemisia absinthium* L. using *Agrobacterium* strain C58C1 had 100% transformation efficiency for one week old explants and the efficiency gradually decrease for two and three week old explants at 90% and 70%, respectively. More over, Mazumdar et al. (2010) reported that for jatropha, the youngest explant, which was derived from cotyledonary leaf of germinated seed showed the greatest response towards transformation as compared to one- and two-week-old explants. Song et al. (2019) also showed that juvenile leaves of hybrid poplar (*Populus alba* × *Populus grandulosa* Uyeki) yield better transformation efficiency than mature leaves when they were subjected to *Agrobacterium*-mediated transformation.

The medium composition has also been found to play a fairly significant role in *Agrobacterium*-mediated transformation. Supplementation of 3,5-dimethoxy-4-hydroxyacetophenone (acetosyringone) specifically has been shown by various researchers to enhance transformation efficiency in different species of plants. Sheikholeslam and Weeks (1987) showed that even a very low concentration of 20 µM of acetosyringone was enough to boost the transformation efficiency of *Arabidopsis* from below 3% up to more than 50%. However, acetosyringone does not

necessarily increase transformation rate of all plant species as was shown by Godwin et al. (1991) whereby using *A. tumefaciens* strain A281, transformation efficiency of *Antirrhinum majus* decreased when 200 µM acetosyringone was supplemented as opposed to medium without acetosyringone. Another study by Sawant et al. (2018) showed that using 11 different concentrations of acetosyringone with 50 µM increments up to 500 µM, the highest transformation efficiency for *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.) calli was obtained when 350 µM of acetosyringone was used. Concentrations above and below this value all resulted in lower transformation efficiency with the lowest being those without acetosyringone supplementation. On the other hand, Ashrafi-Dehkordi et al. (2021) reported that supplementation of 200 µM acetosyringone showed the highest transformation efficiency in the production of transgenic bread wheat (*Triticum aestivum* L.).

Calcium, usually in the form of calcium chloride in media, also play a significant role in altering *Agrobacterium*-mediated transformation efficiency. Omission of calcium from various stage of transformation has been found to enhance the efficiency of transformation. Montoro et al. (2000), for instance, reported that the omission of calcium chloride from the preculture medium prior to co-cultivation significantly increased transformation rate in *H. brasiliensis.* Subramaniam and Rahman (2010) reported similar observation with orchids when calcium was omitted from the co-cultivation medium.

2.3.2(b) Screening of transformants and confirmation of transformation

In order to screen for transformants following transformation, reporter genes are employed. Reporter genes allow for screening of transformants as they encode for