TISSUE CULTURE AND BIOLISTIC –MEDIATED TRANSFORMATION OF *IMPATIENS BALSAMINA*

AISHAH MOHD TAHA

UNIVERSITI TEKNOLOGI MALAYSIA

ABSTRACT

Biolistic- mediated transformation is an approach to transfer a gene of interest into a plant cell using particle bombardment method. Tissue culture experiment was conducted to optimize shoot regeneration from cotyledon explant of 7 day-old seedlings *I. balsamina*. The maximum average number of shoots (6.8 ± 1.05 shoots) per explant was obtained on full strength MS-based medium containing 1 mg/L BAP after three weeks of culture. For rooting induction, there was no significant difference on percentage of root regeneration on full strength (84%-94%) and half strength MS–based medium (78%-96%) supplemented with IAA (0.1 - 1mg/L), IBA (0.5 - 2 mg/L) and NAA (0.1 - 1 mg/L) after two weeks of culture. Plantlets were successfully produced on full strength MS plates supplemented with 1 mg/L BAP (93%) for shooting and half strength MS media supplemented with 0.1 mg/L IAA (92%) for rooting in eight-week culture. Biolistic technique was used for the transformation of *uidA* and *hph* genes into *I. balsamina*. The 7 day- old cotyledons explants were bombarded with pRQ6 containing *uidA* gene encode for *ß*-glucuronidase (GUS) and *hph* gene conferring resistance to hygromycin and co-transformation was carried out using pRQ6 with pAHG11 (contain *bar* gene conferring resistance to herbicide Basta). The physical and biological factors of bombardment such as target distance (6 - 12 cm), helium pressure (650 - 1100 psi), number of bombardments (once and twice), DNA concentrations (0.5 - 1.5µg), pre-culture times (4 - 32h), osmotic treatments using mannitol , sorbitol and combination of mannitol and sorbitol at 0.2 M, 0.4 M and 0.6 M and post-bombardment incubation times (4 - 48 h) were optimized. Transformation of *I. balsamina* with pRQ6 and co-transformed with pRQ6 and pAHG11 at 28 mm Hg vacuum using optimized bombardment conditions (9 cm target distance, 1100 psi helium pressure, one time bombardment, 1.0 µg DNA, 16 h pre-culture time with osmotic treatment of 0.4 M mannitol and sorbitol and 24 h post-bombardment incubation time) showed the highest average number of GUS spots of 149.3 and 128.1, respectively. Delay selection method was used to delay the timing of selection on shooted explants after bombardment to obtain transformed plants. Out of 160 bombarded explants with pRQ6, only 84 plants were successfully regenerated from selected 35-day old shooted explants after five weeks grown on MS –based medium containing 75 mg/L hygromycin. All regenerated plants (84 plants) were GUS positive. However, there was no plant regenerated on MS-based medium containing 1 mg/L phosphinothricin (PPT) after co-transformed with pRQ6 and pAHG11. Furthermore, PCR results showed only 14 of 40 GUS positive plants survived at 75 mg/L hygromycin were *hph* gene positive. In conclusion, the PCR results also showed the possibility integration of *hph* gene into the genomic DNA of transformed plants.

ABSTRAK

Transformasi melalui biolistik adalah satu cara untuk pemindahan gen yang dikehendaki ke dalam sel tumbuhan menggunakan kaedah penembakan partikel. Eksperimen kultur tisu dilakukan untuk mengoptimumkan penumbuhan pucuk daripada kotiledon eksplan *I. balsamina* selepas 7 hari. Maksimum purata pertumbuhan pucuk yang tertinggi (6.8 ± 1.05 pucuk) per eksplan didapati dalam kepekatan media MS penuh yang mengandungi 1 mg/L BAP selepas tiga minggu dikultur. Untuk pertumbuhan akar, tiada perbezaan yang signifikan pada peratusan pertumbuhan akar yang dihasilkan menggunakan kepekatan MS yang penuh (84%-94%) dan setengah kepekatan MS (78%-96%) dengan menggunakan IAA (0.1 - 1 mg/L), IBA (0.5- 2 mg/L) dan NAA (0.1 - 1 mg/L) selepas dua minggu dikultur. Anak pokok yang berjaya dihasilkan dalam kepekatan media MS penuh dengan 1 mg/L BAP (93%) untuk pertumbuhan pucuk dan setengah kepekatan MS dengan 0.1 mg/L IAA (92%) untuk pertumbuhan akar dikultur selama lapan minggu. Teknik biolistik digunakan untuk transformasi gen *uidA* dan *hph* ke dalam *I. balsamina*. Kotiledon eksplan yang berumur 7 hari ditembak dengan plasmid pRQ6 yang mempunyai gen *uidA* untuk *ß-glucuronidase* (GUS) dan gen *hph* yang rintang kepada hygromycin dan ko-transformasi dengan plasmid pRQ6 dan pAHG11 (mempunyai gen *bar* rintang ke atas herbisid Basta). Faktor fizikal dan biologikal seperti jarak sasaran (6 - 12 cm), tekanan helium (650 - 1100 psi), bilangan penembakan (sekali dan dua kali), kepekatan DNA (0.5 - 1.5 µg), masa kultur (4 - 32 jam), rawatan osmotik menggunakan mannitol, sorbitol dan kombinasi mannitol dan sorbitol pada kepekatan 0.2 M, 0.4 M dan 0.6 M dan masa pengeraman eksplan selepas penembakan (4 - 48 jam) telah dioptimumkan. Transformasi *I. balsamina* dengan pRQ6 dan ko-transformasi dengan pRQ6 dan pAHG11 pada 28 mm Hg vakum menggunakan penembakan yang optimal (9 sentimeter jarak sasaran, 1100 psi tekanan helium, 1.0 µg DNA, 16 jam masa kultur dengan rawatan osmotik 0.4 M mannitol dan sorbitol dan 24 jam masa pengeraman eksplan selepas penembakan) menunjukkan purata bilangan bintik GUS yang tertinggi dengan 149.3 dan 128.1, masing-masing. Kaedah penangguhan pemilihan digunakan untuk menangguhkan masa pemilihan pada eksplan yang mempunyai pucuk selepas penembakan bagi mendapatkan tumbuhan yang telah ditransformasikan. Daripada 160 eksplan yang ditembak dengan pRQ6, hanya 84 tumbuhan berjaya ditumbuhkan daripada penangguhan pemilihan 35 hari eksplan yang mempunyai pucuk selepas 5 minggu tumbuh dalam media MS mengandungi 75 mg/L hygromycin. Kesemua 84 tumbuhan adalah positif GUS. Walaubagaimanapun, tiada penumbuhan dalam media MS yang mengandungi 1 mg/L phosphinothricin (PPT) selepas ko-transformasi dengan pRQ6 dan pAHG11. Keputusan PCR menunjukkan hanya 14 dari 40 tumbuhan yang ditumbuhkan dalam 75 mg/L hygromycin adalah positif gen *hph*. Kesimpulannya, keputusan PCR juga menunjukkan kemungkinan integrasi gen *hph* ke dalam genom DNA tumbuhan yang ditransformasi.

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CHAPTER 1

General Introduction

1.1 *Impatiens balsamina* background

I. balsamina is a plant from Asia, North America and South Africa or known as 'Keembung', Garden balsam or Touch Me Not. The name of this plant refers to the elasticity of the valves of the seedpods, which discharge the seeds when ripe. These plants have thick stems and light green leaves with wide range of colors including rose red, rose purple, white and pink (Figure 1.1). The plant is an annual and easily grown in evenly moist, an organically rich, well drained soil in full sun to part shades.

The photoperiodic studies on growth and development of *I. balsamina* have been reported by Nanda and Krishnamoorthy (1967). It was found that while floral bud of this species are initiated with three short day cycles, at least eight such cycles are required for flowering. The number of floral bud and open flowers bear a linear relationship with number of short day cycles. The induced floral buds revert to

vegetative growth unless the plants receive a minimum number of short day cycles needed for flowering and this reversion occurring in a basipetal direction.

This plant also has properties and action according to indigenous medical system. For example, the flowers as cooling agent for external application to burns and scalds and posses marked antibiotic activity against some pathogenic fungi *Aspergillus flavus* and *Candida albicans* (Lee *et al*., 1999). Lawsone-forming multi enzyme complex from *I. balsamina* root cultures contain lawsone, an antimicrobial naphthoquinone. It was found that the root cultures of *I. balsamina* could produce a number of natural products, mostly belonging to the chemical groups of coumarins and napthoquinones including lawsone and Me-lawsone (Figure 1.2) (Eknamkul, 1999).

Figure 1.1 *I. balsamina* with white and purple flower colors. Bar= 0.5m

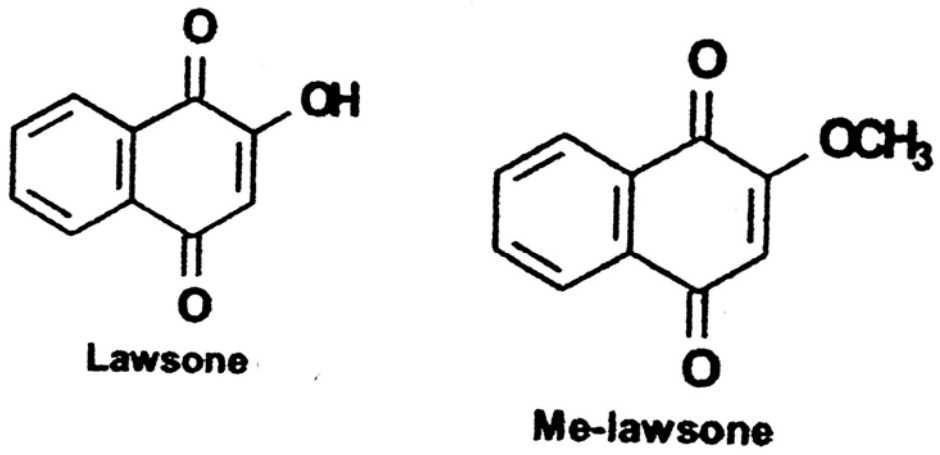


Figure 1.2 Lawsone and Me-lawsone chemical structures, found in roots cultures of *I. balsamina* (Eknamkul, 1999).

1.2 The importance study of *I. balsamina*

The inflorescence study in flower development using *I. balsamina* was reported by Pouteou *et al.,* (1998). This can be explained by the response of axillary meristem primordial to the quantity of inductive signal, a response that is conditioned by the age or position of the primordia and allows undifferentiation of axillary primordial initiated before evocation to adopt different fates. The interpretation of this study is that vegetative and reproductive phase are not separate and antagonistic but interpenetrate each other to varying extends depending on the quantity of inductive signal.

The research on role of the leaves in flower development using *I. balsamina* has been reported by Tooke and Battey (2000). In this research, the leaf derived signal was found to have a role in floral maintenance. The increased petal number was neither a response to a stress affects associates with leaves removal nor a result of alternation of petals for stamen. Rather, the petal initiation phase was prolonged when the amounts of a leaf derived signal were limiting. Therefore, leaf derived signal has a continuous and quantitative role in flower development. This may provide an explanation for the wide variety and instabilities of floral form seen among certain species in nature.

The research on *Impatiens* was also carried out for *Impatiens* Necrotic Spot Virus. Over the past decades, the virus began to develop in floriculture industry and becoming one of the most important viral pathogens of floral crops. More than 300 species from 50 plants families were susceptible to *Impatiens* Necrotic Spot Virus including *Impatiens*. Symptoms of virus including ring spots, brown to purple on the

leaves and stem and flower breaking of plants (Windham *et al*., 1998). To date, there are few reports of genetically engineered disease resistance in floral crops including the transformation using nucleocapsid gene of Tomato Spotted Wilt Topovirus (TSWT) into chrysanthemum plants (Daughtery *et al*., 1997). Baxter (2005) also reported on development of *Impatiens* Necrotic Spot Virus resistant using the nucleocapsid gene to *I. wallenaria* via *Agrobacterium*-mediated transformation.

1.3 Plant regeneration system

Plant regeneration system could be achieved via tissue culture. Tissue culture is the culture and maintenance of the plant cell or organs in sterile and environmentally supportive condition *in vitro*. It also refers as micropropagation, which is a set of techniques for the production of whole plants from cell cultures, derived from explants of meristem cells. Plant cells do need to multiply *in vitro* in certain condition and the most important is the freedom of the cells from competition of other organ. Therefore, it is necessary to remove the contaminants from the culture in aseptic conditions for the plant cells to multiply and produce the whole plant. This is usually conducted by surface sterilization of the explants with chemicals such as bleach and mercury chloride at the certain concentration and duration that killed or removed the pathogens without injuring the plant cells. Sterilizations of medium and apparatus used and maintenance the sterile condition while culturing the explants also need to be done to avoid the pathogens (Lineberger, 2003).

The successful of plant cell cultures is depended on the type of media (Murashige and Skoog, (1962); Gamborg B5) containing macro element, micro element, iron and vitamins supplemented with appropriate hormones for the growth. By providing the necessary chemicals in appropriate combinations and forms, it has been possible to establish cultures from virtually every plant part, of plants ranging from mosses to monocotyledonous angiosperms and to pursue diverse academic and economic aims. The ingredients of plant culture media can be categorized as inorganic salts, organic compounds, complex natural preparations and inert supportive materials (Huang and Murashige, 1976).

Tissue culture has wide applications in research as it offers numerous benefits over the traditional propagation methods. For example, a single explant can be multiplied into several thousand plants in less than one year. Once established, actively dividing cultures are a continuous source of microcuttings, which can result in plant production under greenhouse conditions without seasonal interruption. The rate of growth is much faster due to the addition of exogenous hormones *in vitro* compared with traditional methods. The environment in culture provides nutrient and sterile conditions to avoid from other organism and the competitor of nutrient uptake (Lineberger, 2003; Slater *et al*., 2003).

Example of plant regeneration of *Impatiens* reported were *I. wallenaria* (Baxter, 2005), *I. platypetala* (Kyungkul, 1993), *Impatiens* L. interspesific hybrids T63-1, a Java (J) x New Guinea (NG) (Kyungkul and Stephens, 1987) and Java, New Java and Java x

New Guinea (Stephens, 1985). Till now, no plant regeneration of *I. balsamina* is reported.

1.4 Transformation system

Transformation is the introduction of foreign DNA into the plant cell by means of *Agrobacterium tumefaciens* and biolistic method. According to Birch (1997), the first plant transformation was reported in 1984 and has been extended to over 120 species in about 35 families. Plant transformation research has been widely provided as the expectations of this approach in improving the quality, to obtain the high yield and varieties resistant of plants (rice, orchid, soybean and pepper) against herbicides and diseases. The plant transformation remains as an alternative used to enhance the productivity and quality of plants. This improvement could lead for the future prospect, as the novel genes can be introduced for the useful production ranging in industrial, economical and agricultural product.

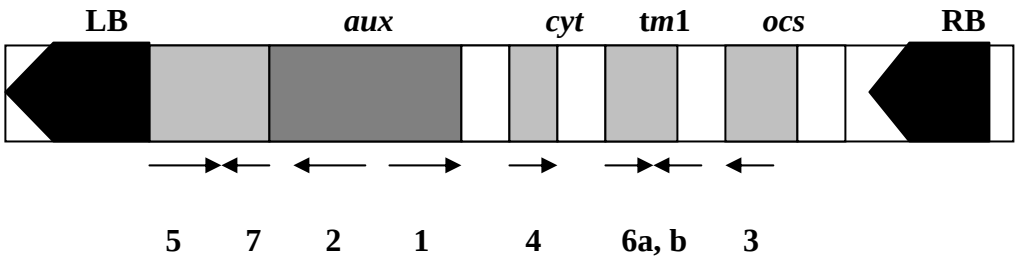
The conventional breeding limited the high yield of plants, as it is a time consuming process to develop a variety of desired plants. The major limitation of conventional methods derive from the limitations of the sexual process itself and include constrains on the amount of genetic variation available within the crop (the gene pool) and the fact that all traits differing between the parents are subject to segregation and thus of selection are required to identify rare individuals that combine the best qualities of both parents (Manshardt, 2004).

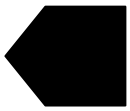
The most widely successful systems used for plant transformation are biolistic and *A. tumefaciens* mediated transformation system. Biolistic is one of the methods that used high velocity microprojectiles to penetrate into the cells. This method depends on the apparatus optimization to enhance the transformation. In contrast, *Agrobacterium* technique uses the biological approach of plant pathogenic bacteria that moves the DNA from its plasmids into the plant cells as part of its life cycle (Slater *et al*., 2003).

1.4.1 *Agrobacterium tumefaciens* transformation

The *A. tumefaciens,* the causative agent of crown gall disease is a Gram negative bacterium, rod in shape, found in rhizophere and survives on plant nutrient. This bacterium can infect the wound site of wounded plants and cause tumors. *A. tumefaciens* attracted to the wound site via chemotaxis shows the reaction to the phenolic compounds released from the damage cell. The Ti plasmid region of *A. tumefaciens* containing the T-DNA is defined as the presence of right (TR-DNA) and left borders (TL-DNA). Any DNA inserted between these borders will be transferred into the genome of the plant host and this system has been used to introduce the desired gene into plant during transformation. However, only the TL-DNA is oncogenic and contains eight open reading frames. The organization of the genes in the TL- DNA is shown in Figure 1.3 (Slater *et al*., 2003). The TR-DNA can be transferred and maintained in the absence of TL-DNA. Therefore, TR-DNA is considered as a naturally occurring T-DNA vector which might be an alternative to manipulated TL-DNAs from which all *onc* genes have been deleted (Leemans *et al*., 1983).

Although the used of *A. tumefaciens* has been widely reported in transformation of dicotyledonous, there were reports of the use of biolistic as an alternative method to enhance the expression and incorporation of genes into the plant cell in dicotyledonous species. For example, the first report describing recovery of an intact transgenic plant soybean (*Glycine max*) using biolistic was published (Christou, 1994). However, there was no report on transformation work of *I. balsamina* using *A. tumefaciens* and biolistic methods.





Border repeat (LB=left border, RB=right border).

Figure 1.3 The genetic organization of the TL-DNA of an octopine – type Ti plasmid with eight open reading frames (ORFs) (1-7) (Slater *et al*., 2003).

1.4.2 Biolistic- mediated transformation

Biolistic is one of the methods used for genetic transformation. Many transgenic plants have been produced via this approach. The first generation of this technique for gene transfer into plant cells was developed in 1960s by plant virologist (Birch and Bower, 1994). Transgenic plants generated by this method have been reported for monocotyledonous such as Kentucky bluegrass (Gao *et al*., 2005), jute (Ghosh *et al*., 2002), rice (Jain *et al*., 1996; Li *et al*., 1993) and dicotyledonous species including mothbean (Kamble *et al*., 2003), cowpea (Ikea *et al*., 2003), sunflower (Molinier *et al*., 2002) and soybean (Moore *et al*., 1994).

This method involves the use of a high velocity of particles to penetrate the cells and introduce the DNA into the cells. The introduced DNA will be expressed in the plant cell if it is into plant chromosomal DNA (Wong, 1994). The particles that are commonly used are gold and tungsten. For biolistic transformation, DNA is coated onto the surface of gold or tungsten by precipitation with calcium chloride and spermidine. Generally with the high velocity, the DNA was delivered into the cells and once inside the cells, the DNA will elute off. If the foreign DNA reaches the nucleus, then transient expression will likely result and the transgene may be stably incorporated into host chromosomes (Kikkert *et al*., 2003; Birch and Bower, 1997).

One of the widely used device for biolistic transformation is PDS-1000/He Particle Delivery System (Figure 1.4). This instrument used the high pressure helium

which carries the most power for given pressure of compressed gas without danger of explosion associated with hydrogen compared to other gases, with the less shock wave (Wong, 1994).

One of its components is the rupture disc. The rupture disc determines the acceleration of shock wave to deliver the particle and available in different thicknesses ranging from 450 psi to 2200 psi. The rupture disc sealed the gas tube and released the shock wave when the valve is opened as the helium gas flows into the gas tube and pressuring the cavity. The energy of shock wave will accelerate the particles to the target cell. The distance of the target distance can be adjusted at different positions (3 cm, 6 cm, 9 cm and 12 cm) from the stopping screen. This target distance determines the spread of the particles to the target cell. The macrocarrier disk is held by the metal ring and the particles coated with the DNA were spread on the surface of macrocarrier. The stopping screen and macrocarrier sheet are subjected to the violent forces during operation. The operation is held under vacuum to help the blast of helium shock wave (Wong, 1994).

The physical parameters depend on the apparatus settings. Generally, the wide ranges of settings have been tested (target distance, helium pressure and number of bombardments) and show the optimal for most of plants including banana (Sreeramanan *et al*., 2005), wheat (Ingram *et al*., 1999) and rice (Jain *et al*., 1996). The use of gold particle in range 0.7 μm to 1.0 μm, vacuum of 28 Hg, helium pressure

Figure 1.4 PDS-1000/He Particle Delivery System, a device used in biolistic transformation (BioRad, USA).

of 1100 psi and gap distance of 6 cm to 10 cm result in high transformation rates (Kikkert *et al*., 2003). For example, biolistic-mediated transformation on callus of rice resulted on transformation frequency with 37.5% (Ramesh and Gupta, 2005) and 8.9% (Lee *et a*l., 2003).

The necessity to optimize the velocity using the target distance and helium pressure is for optimal transformation rates with different tissues types, depending on the cell wall thickness and the need to penetrate several layers (Birch and Bower, 1994). For example, target distance of 9 cm and 1100 psi helium pressure in single bud of banana (Sreemanan *et al*., 2005), immature embryos of sorghum (Tadesse *et al*., 2003), embryos of cowpea (Ikea *et al*., 2003) and in embryogenic cells of rice (Jain *et al*., 1996) was the optimal condition in biolistic-mediated transformation.

Multiple bombardments are carried out with the objective of getting better coverage of targeted areas in banana tissues (Sreeramanan *et al*., 2005). This is in consensus with Ikea *et al.,* (2003) who reported that bombarding the embryos of cowpea twice increased the number of GUS gene expression with 70% of shoots embryos showed more than 20 blue spots per embryos hit and this could be due to the fact that multiple bombardments allow better coverage of the target areas and compensate for misfires from faulty and poorly set rupture discs.

The biological factor includes the physiological condition of cell such as cell turgor pressure. The osmotic treatment with stabilizer sugar such as mannitol and

sorbitol can increased the transformation rates. The mechanism of osmotic enhancements could include both reduced vacuole volume and reduced turgor, which would alter penetrability by particles (Birch and Bower, 1997). It has been proposed that osmotic treatment could prevent the damages of cell membrane and loss of cytoplasm. In addition, the reduction of the volume of the vacuoles increased the possibility to reach the nucleus, resulting in larger number of cells successfully expressing the introduced gene (Santos *et al*., 2002). Transformation efficiency is also affected by subjecting the bombarded tissue at the right stage to the GUS assay for the recovery of the cells from injuries caused by bombardment (Sreeramanan *et al*., 2005).

Other biological parameter includes the DNA concentration, tissue type, cell culture age and mitotic age (Wong, 1994). However, increasing the concentrations of DNA precipitated will also increase transient expression frequencies until particle aggregation occurs, resulting in poor dispersal and increased cell damage (Birch and Bower, 1994). On the other hand, the exogenous DNA used in the transformation experiments typically comprises a plant expression cassette inserted in a vector based on the high copy number bacterial cloning plasmid. Neither of these components is required for DNA transfer and only the expression cassette is required for transgene expression. The expression cassette typically compromises a promoter, open reading frame and polyadenylation site that are functional in plant cells. Once this plasmid has been isolated from the bacterial culture, it is purified and used directly as a substrate for transformation (Altpeter *et al*., 2005).

Therefore, the application of particle bombardment has been wide in transient gene expression studies. The transient expression study which initiates the genetically transformed plants enable for the study of mutations, promoter, and the effect of gene expressions (Taylor *et al*., 2002). In contrast, major disadvantage of biolistic is the tendency for complex integration patterns and multiple copy insertion could cause gene silencing (Kikkert *et al*., 2003).

The application of biolistic has demonstrated transient gene expression in plant studies, production of genetically transformed plant and tissue and inoculation with viral pathogens (Taylor *et al*., 2002). The universality of application through cell types, size, shape, presence or absence of cell wall, and direct introduction of biological material into the cell have very high delivery efficiency to enhance the transformation rates (Sanford *et al*., 1987).

1.5 Marker genes

1.5.1 Selectable marker

The used of selectable marker genes is to identify those cells that successfully integrate and express the transferred DNA. Genes conferring resistance to various antibiotics or herbicides such as hygromycin and phosphinothricin (PPT) respectively are commonly used in transformation research. The genes (*hph* gene and *bar* gene)

encode proteins that detoxify corresponding selection agents and allow the growth of transformed cells (Goodwin *et al*., 2003; Slater *et al*., 2003).

1.5.1.1 Antibiotic

Hygromycin B is an aminocyclitol antibiotic with broad spectrum activity against prokaryotic and eukaryotic cells by interfering with protein synthesis (Waldron *et al*., 1985; Rao *et al*., 1983). Hygromycin B appears to interfere with amino acyl t-RNA recognition and cause misreading in cell free polypeptide synthesizing system that could prevent the synthesis of protein needed for the growth of cells (Rao *et al*., 1983).

The resistance can be conferred by *hph* gene encoding *hygromycin phosphotransferase*, which was originally found in the strain of *E.coli* (Waldron *et al*., 1985). Resistance to hygromycin B is determined by an *aminocyclitol phosphotransferase* that modifies hygromycin B. The specific modification of hygromycin B is a phosphorylation of the hydroxyl on the 4 position of the cyclitol ring (hyosamine) (Rao *et al*., 1983) as showed in Figure 1.5.

The hygromycin B resistant plants have been developed for both monocots and dicots such as rice with 8.9% transformation frequency (Lee *et al*., 2003), Kentucky bluegrass with 77.8% transformation frequency (Gao *et al*., 2005) and orchids with 12% transformation frequency (Men *et al*., 2003).

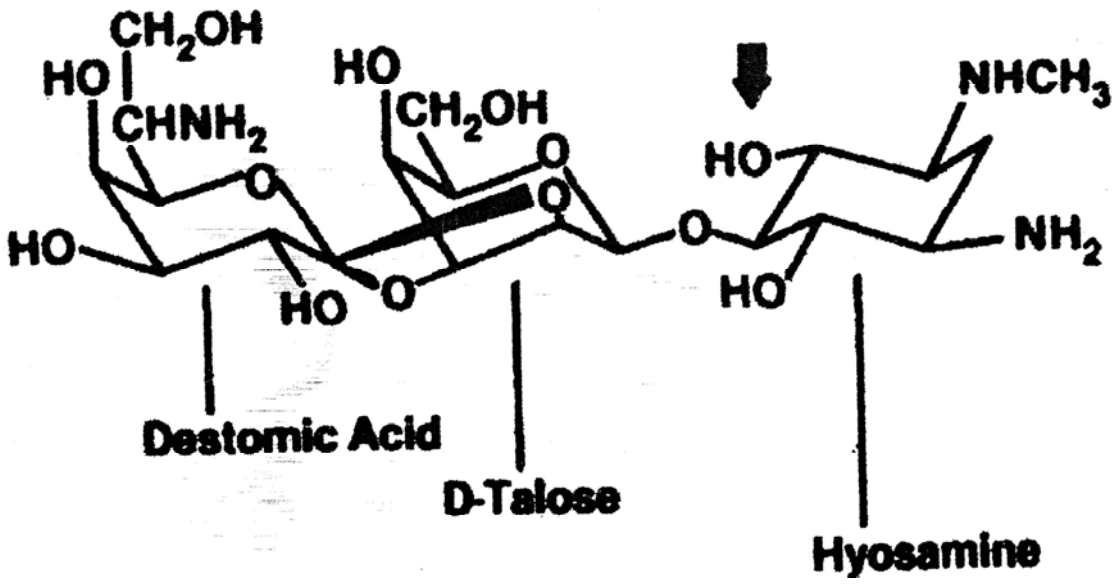


Figure 1.5 Structure of hygromycin. The arrow indicates the 4-hyroxyl group which is the site of phosphorylation for inactivation of hygromycin (Rao *et al*., 1983).

1.5.1.2 Herbicide

Weeds are one of the major problems encountered in crop management because of competition with crops for water, nutrients and as result decrease farming yields and productivity. Herbicide resistant crops are planted and non- selective (broad spectrum) herbicides are used for weed management. Provided that the field crops are genetically modified the carry gene for herbicide resistance, the broad spectrum herbicides (glufosinate and glyphosate) will not harm the resistant crops (corn, soybean, sugar beet,

carnation, flax, tobacco, rice and wheat). The other herbicides used are bromoxynil, sulfonamides and sulfonylurea (Mayer *et al*., 2004).

Phosphinothricin (PPT) is also known as glufosinate, one of the active ingredients of herbicide Basta. It belongs to the family of aminoalkylphosphonic acids, structural analogues of amino acids in which the carboxyclic group is replaced by a phosphonic. This group resembles the tetrahedral transition state of several enzymatic reactions, particularly amide bond formation and hydrolysis. Several enzymes unable to discriminate between carboxylic and phosphonic function for binding to active sites, therefore results in inhibition of enzyme activity (Guiseppe *et al*., 2006).

Phosphinothricin (PPT) acts by inhibiting the glutamine synthetase, a key enzyme in nitrogen metabolism in bacteria and plant cells (Berlicki *et al*., 2006; Wolfgang *et al*., 1994). This rapidly leads to ammonium accumulation and lack of glutamine. Glutamine synthetase catalyzes a reaction of central importance in plant metabolism, the conversion of glutamate to glutamine (Eugene *et al*., 1991). The enzymes catalyze the conversion of glutamate to glutamine in the presence of ammonium ion with accompanied hydrolysis of adenosine triphosphate (ATP) as an energy source. Inhibition of this enzyme in plants causes total impairment of nitrogen metabolism resulting in accumulation of toxic amount of ammonium, which followed by plant death (Giuseppe *et al.,* 2006).

Being nonselective herbicides, the exposure of phosphinothricin (PPT) to the plant may also block the recycle of carbon from the photorespiratory pathway to the Calvin cycle (Eugene *et al*., 1991). The genetic engineering of phosphinothricin (PPT) resistant plants allows the enlargement of the application of the herbicide in transgenic plant (Wolfgang *et al*., 1994). Figure 1.6 shows structure of phosphinothricin (PPT).

The bialaphos (*bar)* resistance gene of *Stereptomyces hygroscopicus* that encodes for phosphinotricin acetyltransferase, which confers resistant against Basta has been widely used in transformation research (Ghosh *et al.*, 2002). The *bar* gene converts phosphinothricin (PPT) into nontoxic acetylated form and allows growth of transformed plant even in the presence of phosphinothricin (PPT) (Goodwin *et al*., 2003).

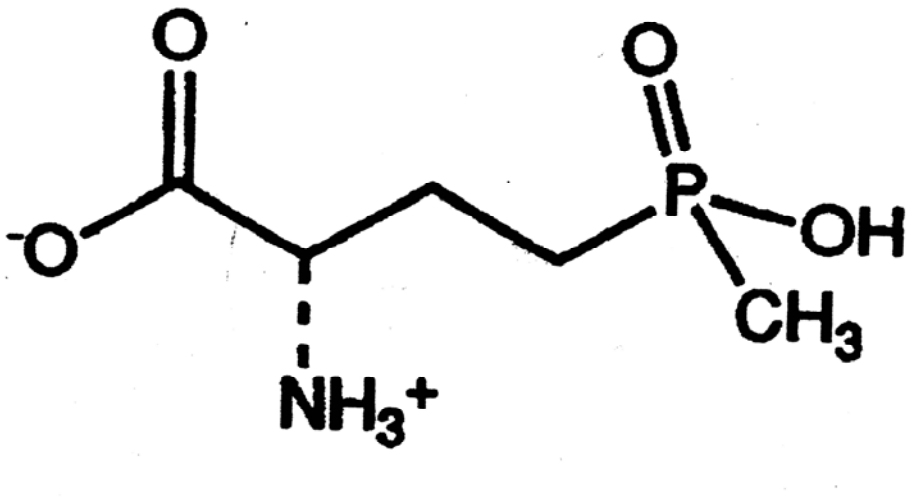


Figure 1.6 The chemical structure of phosphinothricin (PPT) (Eugene *et al*., 1991).

1.5.2 Reporter genes

The most convenient measure of efficiency for DNA delivery into intact cells is the number of cells, which transiently express incoming reporter gene. Reporter genes or reporter proteins have played an important role in developing and optimizing transformation protocols for plant species (Sreeramanan *et al*., 2005). Reporter genes should be easy to assay, preferably with a non-destructive assay system and there should be little or no endogenous activity in plant to be transformed (Slater *et al*., 2003).

Reporter genes codes for enzymes or protein can be detected using the biochemical assay. The efficient reporter gene allows the detection of transgenic events after bombardment in either a transient or stable expression. The most commonly used reporter gene is *uidA* gene encoding the ß-glucuronidase (GUS). Other reporter genes are green fluorescent protein (gfp), chloramphenicol transferase (CAT), nopaline syntase (NOS), octopine syntase (OCS) and luciferase (Datta *et al*., 1997).

The GUS gene, originally isolated from *E.coli* has been widely used in transient expression study and plant transformation as the stability of the enzymes and the high sensitivity of the GUS allowed qualitative and quantitative assessment of transgene expression levels (Cervera, 2003; Taylor *et al*., 2002; Jefferson *et al*., 1987).

Various ß–glucuronide acid substrates are available for GUS expression, which contained the D-glucopyranosiduronic acid attached glycosidic linkage to a hydroxyl

group of detectable molecule. The preferred substrate for detection is 5-bromo-4-chloro-3-indoxyl-ß-D-glucuronide (X-Gluc) (Datta *et al*., 1997). After bombardment, the explants were incubated with GUS histochemical substrate, 5-bromo-4-chloro-3-indoxyl-ß-D-glucuronide (X-Gluc) (Kikkert *et al*., 2003) (Figure 1.7). Those cells that express GUS activity stained blue and distribution of cells received and expressed the bombarding DNA is easily visualized (Jefferson *et al*., 1987). However, disadvantage of using GUS is the blue stain is toxic and the assay therefore resulting in destruction of GUS expressing cells (Birch and Bower, 1994).

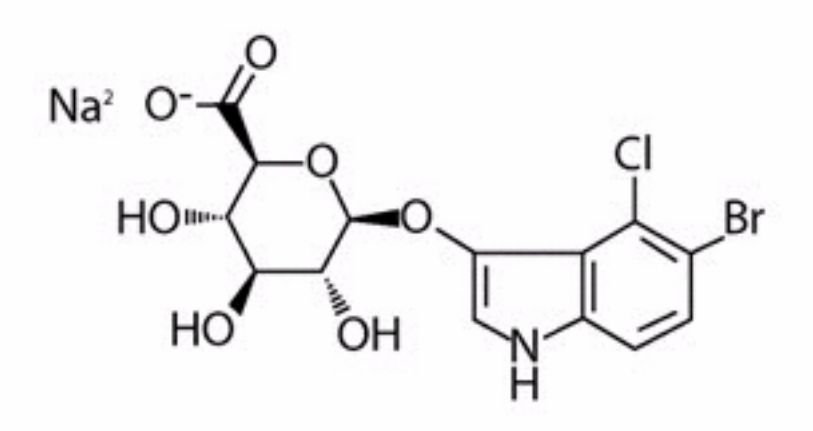


Figure 1.7 Structure of X-Gluc (5- bromo 4-chloro-3-indoxyl-ß-D-glucuronide, a substrate for GUS which is encoded by *uidA*, a widely used reporter gene (BioWorld, USA).

1.6 Transient gene expression and stable gene expression

Generally, there are two types of gene expression in transformation. The transient gene expression is temporary, occurs almost immediately after gene transfer with the higher frequency than stable integration and does not require the regeneration of whole plants. Therefore, transient expression is a rapid and useful method for analyzing the function of gene of interest (Altpeter *et al*., 2006; Lincoln *et al.,* 1998) and the transient expression frequency provides the most convenient measure of the frequency of introduction of DNA into explants during the optimization of bombardment conditions (Birch and Bower, 1994). In contrast, although the stable expression occurs with the lower frequency, the expression was maintained for long term as the DNA incorporate into the chromosome of the recipient cell (Datta *et al*., 1997; Mitrovic, 2003).

Introduction and expression of an exogenous gene into cells does not always involve stable integration of the gene into the genome of the recipient cell. Frequently, plasmid DNA may be introduced into host cells and may express in a transient fashion. This activity declines over time and eventually disappears. Even though measuring levels of such transient activity may be useful in specific cases, when it comes to the creation of stable transgenic phenotypes, only transformation events leading to integration of the foreign gene into the genome of the host cell are useful (Christou, 1994).

1.7 Promoters

According to Odell *et al.,* (1985), the cauliflower mosaic virus (CaMV) promoter *35S* is a major promoter of cauliflower mosaic virus that infects member of *Cruciferae*. The promoter is well characterized and stronger than other plant promoter expression in dicots, but confers low level of expression in monocots. The rice actin promoter and maize ubiquitin promoter achieved far better expression compared to *35S* promoter in most monocots tested. The cauliflower mosaic virus (CaMV) fragments containing 400 to 1000 base pairs of *35S* upstream sequences have been shown to be active when integrated into nuclear genome of transgenic tobacco. The upstream fragment -343 to -46 is responsible for the majority of the *35S* promoter strength. The upstream fragments can be subdivided into three functional regions, -343 to -208, -208 to -90, and -90 to -46. The first two regions can potentiate the transcriptional activity and the third region itself increasing the transcriptional activity of the first two regions (Figure 1.8) (Fang *et al*., 1989).

The specific sequence (a -60 nucleotide region (S1)) downstream of the transcription initiation site of the cauliflower mosaic virus (CaMV) *35S* can enhance gene expression. By using transient expression assays with plant protoplast, this activity was shown to be at least partially due to the effect of transcriptional enhancers within this region. The sequence motif with enhancers function is identified, which are normally masked by the powerful upstream enhancers of the *35S* promoter. A repeated CT-rich motif is involved both in enhancer function and in interaction with plant nuclear proteins (Pauli *et al*., 2004).

Figure 1.8 Nucleotide sequence of the Cauliflower Mosaic Virus *35S* promoter and upstream regions (Fang *et al*., 1989).

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