2.0 LITERATURE REVIEW

2.1 The aquatic plants

Aquatic plants are the main source of food and oxygen in the water. They are of utmost importance for the maintenance of biological balance in the water ecosystem (Rataj and Horeman, 1977). Besides that, these water plants provided a sense of security when the fishes feel threatened by their natural predators. The water plants can be categorised into 4 groups as summarised in **Table 2.1**.

Water plants are cultivated in the aquarium in order to create an ideal environment that mimic the natural niche for many fishes. They not only used as decorative plants in the aquarium but at the same time facilitate the acidification of the water in the aquarium as the low pH is more favourable for many fishes to survive and spawn (Rataj and Horeman, 1977). Healthy aquarium plants help to improve the water quality and oxygenate the fish tank. Some even contain bactericides that protect the fishes from harmful bacteria.

Categories of the aquatic plants	Characteristic	Example genus
Submersed plants	Rooted at the bottom, flowers and produce seeds under the water.	*Vallisneria*, *Barclaya* and *Ottelia*
Half submerged	Rooted at the bottom in the water with leaves reaching the water surface. Sometimes flowers when half emerge.	*Myriophyllum* and *Heteranthera*
Floating plants	Float and live in the water surface. Roots float freely in the water. Flowers are always on the surface and pollinated in the air.	*Lemna*, *Limnobium*, *Utricularia* and *Eichhornia*
Amphibious plants	Grow submerged or emerged in the water, land form arise when the water dried out occasionally.	*Echinodorus*, *Sagattaria*, and *Cryptocoryne*.

Table 2.1: Categories of aquatic plants

Source: Rataj and Horeman (1977)

2.2 The *Cryptocoryne* species

The genus *Cryptocoryne* belongs to the family of Araceae, comprising 60 species mainly found in tropical Asia mainly India, Peninsula Malaysia, and Asiatic islands. These areas are mainly covered by jungle with small lakes, rivers and marshes which provided nearly constant high temperature (26^{o}C – 28 ^{o}C) for the *Cryptocoryne* species. Furthermore, this group of water plants prefer to live in a place where water has no periodic variation thorough the year (Rataj and Horeman, 1977).

The shape of the petiole and the blade of the leaf are distinctly variable for different *Cryptocoryne* species. The colouration of the leaf also varies according to species type and sometimes to the intensity of light. *Cryptocoryne* that grows in deep shade usually has coloured red to brown leaves and petioles meanwhile green coloured leaves and petioles are predominates in those sun-light flavoured *Cryptocoryne*.

The inflorescences for the whole family of Araceae resemble a trumpet and this gives them the name of "water trumpet" which reflects the shape of their inflorescence. The inflorescence is of the utmost importance for the scientific classification of the *Cryptocoryne* species. However, many species of *Cryptocoryne* rarely flower in artificial condition as well as in the nature.

Most of the time, new plants arise by the means of vegetative propagation from dormant buds or rhizome runners. The *Cryptocoryne* species can propagate vegetatively so rapidly and become predominate endemic species in nature (Rataj and Horeman, 1977).

Cryptocoryne willisii originated from Sri Lanka (Scheurmann, 1987). It is among the small *Cryptocoryne* species that is popular for the aquarist because of the exotic beautiful coloured leaf and ease of cultivation. Its' leaves usually ranged from 4 – 5 cm long, 0.5 cm to 2.5 cm wide (Hiscock, 2005). The leaves are usually painted with red-brown colouration and sometimes with dark stripes that attract the attention of most hobbyist and aquarist.

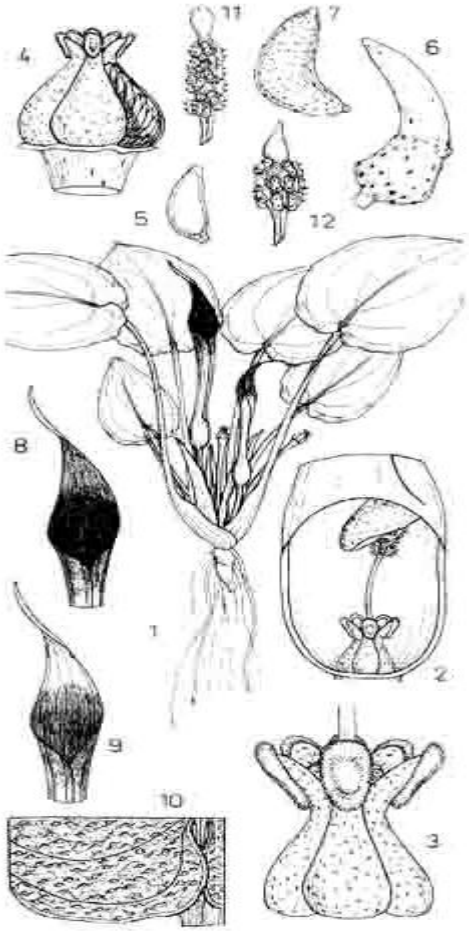

Figure 2.1: Botanical drawing of *Cryptocoryne sp.*

Source: http://www.nationalherbarium.nl/Cryptocoryne/Gallery/wil/wil.html

2.2.1 Cultivation and tissue culture of *Cryptocoryne* species

The *Cryptocoryne* species are popular aquarium plants that are cultivated commercially world wide. **Table 1.2** lists some of the selected *Cryptocoryne* species that are currently available in the aquarium market in Malaysia. *Cryptocoryne* species are usually cultivated at low light intensity, at temperatures of 20^{o}C to 25^{o}C which is the optimum temperature for most of the aquarium fishes to live with.

Cultivation of certain *Cryptocoryne* species is difficult because these plants are sensitive to fluctuation change of temperature. Intense care and prior knowledge are required to ensure successful adoption of the *Cryptocoryne* in cultivation. Furthermore, slow vegetative propagation of the domesticated *Cryptocoryne* in the aquarium (Windelov, 1987) and uncharacterised disease (Ridings and Zettler, 1973) caused supply and quality problems in the production. Hence, tissue culture approach was employed as an alternative propagation technique.

In vitro cultures of aquatic plants were previously reported by Harder (1968), Kukulczanka *et al.* (1980) and Huang *et al* (1994) in their work on *Aponogeton* and *Anubias barteri* species. Meanwhile, micropropagation of *Cryptocoryne* was first reported by Kane *et al.* (1990) on the species of *C.*

lucenswendtii. The same group developed an extended procedure for *in vitro* propagation, auxiliary shoot proliferation and plantlet acclimatization on the other commercially important *Cryptocoryne* species, *C. wendtii* De Wit (Kane *et al.*, 1999).

2.3 Gene transfer to plants

Plant transformation is carried out to facilitate understanding of biological processes in plants and to introduce new traits for improvements (Greenberg and Glick, 1993). Foreign genes have been introduced and delivered into plant cells via direct and indirect gene transfer methods as described in **Section 2.3.1**. Though all of these methods are unique and serves different applications, transformation using *Agrobacterium* and biolistic bombardment are most extensively used and currently prevailed (Dai *et al.*, 2001).

Every plant transformation event must be followed by stable integration of the particular transgene into the plant genome and inherited at the subsequent generations (Gruber and Crosby, 1993). Three key components are crucial and vital in plant transformation system: Firstly, selectable markers and selection condition to rule out the non-transformant. Secondly, regenerable and efficient *in vitro* culture system that ensures a rapid propagation of transformed materials in contamination-free condition. Thirdly, method of delivery that brings minimum damage to the plant cells (Songstad *et al*., 1995).

2.3.1 Methods of gene transfer to plants

2.3.1.1 Direct gene transfer

Direct gene transfer methods such as biolistic bombardment (Sanford, 1988), liposome fusion or polyethyleneglycol – mediated transfer (Uchimiya *et al.*, 1986), microinjection (de la Pena *et al.*, 1987), protoplast and cell electroporation (Fromm *et al.*, 1985) are the methods developed to compensate the host range limitations on monocotyledonous species of *Agrobacterium* and problems of recalcitrancy in some dicotyledonous plants species (Potrykus, 1991).

However, *Agrobacterium* – mediated transformation has remarkably advantages over these direct transformation methods (de la Riva *et al.*, 1998). Its' practice reduces the copy of the transgene inserted which is associated with fewer problems in transgene silencing, cosuppression and instability (Koncz *et al.*, 1994; Stam *et al.*, 1996).

2.3.1.2 Indirect gene transfer – *Agrobacterium* mediated transformation

Indirect gene transfer to plants methods are based on the utilisation of *Agrobacterium*, a soil borne, gram- negative bacterium which is a natural pathogen to dicotyledonous plants. The pathogenicity of *Agrobacterium* to plants varied depending on the species. *A. tumefacies* causes "crown gall" disease in plants (Smith and Townsend, 1907), while *A. rhizogenes* causes "hairy roots" phenomenon in plants (White and Nester, 1980).

Agrobacterium – mediated transformation has been successfully reported in more than 120 species of at least 35 families including the crops of economic importance, vegetables, herbs, fruits, tree, pasture plants as well as the ornamental plants (Birch, 1997). Efficient methodologies have been established for *Agrobacterium* – mediated transformation in dicotyledonous plants which are the natural host range for *Agrobacterium*. In adition, a number of monocotyledonous plants including rice (Hiei *et al*., 1994; Cheng *et al*., 1998) wheat (Cheng *et al*., 1997), maize (Ishida *et al*., 1996), sorghum (Zhao *et al*., 2000) and sugarcane (Enríquez-Obregón *et al*., 1997) have now been transformed with *Agrobacterium*. Moreover, with the advancement of vectors construction and modification, problems faced during *Agrobacterium* transformation of monocotyledonous plant cells have been reduced (de la Riva *et al*., 1998).

2.3.1.3 Ti plasmid of *A. tumefaciens* and the T – DNA

The plant transformation ability of this *A. tumefaciens* lies in the ability of introducing a segment of its tumour – inducing (Ti) plasmid (Hooykaas and Schilperoott, 1992), the transferred DNA (T-DNA) into the plant nuclei where it becomes integrated into the genome of the host plant (Grant et al, 1991).

Ti plasmid of the *A. tumefaciens* is a relatively large plasmid of approximately 200 kilo basepairs (kb). They are classified according to opines such as mannopine, agropine and fructopine which are the metabolic substrates produced by the host plant required by the *Agrobacterium* (de la Riva *et al.*, 1998). The genes for the production of opine are present inside the T-DNA region of Ti-plasmids. Other than the opine synthesis genes, the oncogenic genes also reside inside the T-DNA region of Ti plasmids. Integration of T-DNA borne oncogenes into the plant genomes will results in the crown-gall formation as a consequence of higher exogenous level of plant growth regulators (PGR), the auxin and cytokinin. These PGR stimulate cell divisions that lead to tumour formation.

The T–DNA is flanked by a left border (LB) and a right border (RB) of 25 bp imperfect direct repeat sequences. The consensus sequences of the T–DNA borders for nopaline strains and octopine strains Ti plasmids are shown

as **Figure 2.2**. The LB and RB border sequence is crucial and determines the T–DNA transfer in a polar fashion (Wang *et al*., 1984). Abolishing the first 6 bp or the last 10 bp of the T–DNA border sequence blocks T–DNA transfer (Wang *et al*., 1987). Moreover, this direct repeats also act as a *cis* element or enhancer at the right border (Peralta and Ream, 1985).

Outside the T–DNA region, resides the origin of replication, conjugative transfer region, the virulence (*vir*) genes and the genes that encoded the enzymes for opine catabolism. The opine catabolism genes are transcribed by the crown gall cells producing enzymes that are vital for *Agrobacterium* to utilize opine as a source of carbon and nitrogen (Hooykaas and Schilperoort, 1992).

2.3.1.4 Factors influencing the success of Agrobaterium-mediated transformation

Several factors have known to be significantly influencing the success of Agrobacteium-mediated transformation (Veluthambi *et al.*, 2003). These include infection time, co-cultivation period, density of Agrobacterium, and bacterial strain. The addition of inducer such as acetosyringone is also taken into consideration for optimisation (Yong *et al.*, 2006). Sometimes, types of wounding yield different efficiencies in Agrobacterium-mediated transformation of plant (Aldemita and Hodges, 1996; Dillen *et al.*, 2000).

Preculture of explants on occasion increase susceptibility of plant and thus increase the rate of transformation.

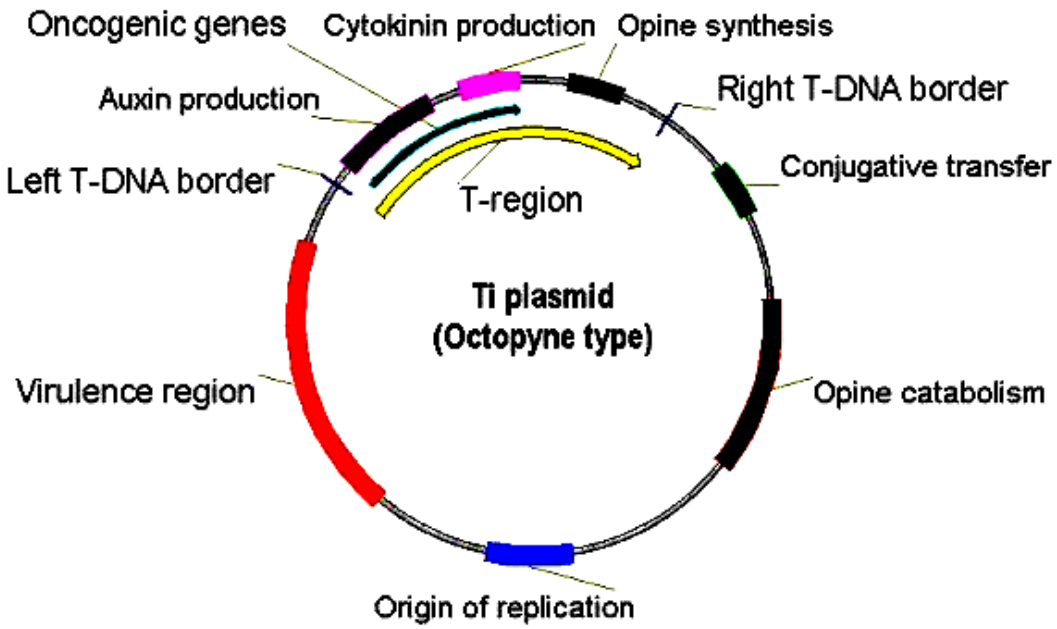

Figure 2.1: General Ti – plasmid map

Source: http://arabidopsis.info/students/paaras/ti_plasmid.jpg

2.3.1.5 The T – DNA transferring machinery and mechanism

The process of T – DNA transfer has been constituted by three genetic elements: one chromosomal element, the chromosomal virulence genes (*chv*) and two elements from the Ti-plasmid itself, the LB and RB and the Ti plasmid virulence genes (*vir*).

The vir genes on the Ti plasmid derived from six operons (*virA, virB, virC, virD, virE* and *virG*) play important roles in transferring T–DNA (Hooykaas and Schiilperoort, 1992; Zupan and Zambryski, 1995; Jeon *et al*., 1998). The *virA, virB, virD,* and *virG* are necessary for T – DNA transfer whilst *virC* and *virE* function in transferring efficiency. Hence, tumour formation was suppressed in strains with mutation in *virC* and *virE* genes (Draper and Scott, 1991). The only constitutive operons, *virA* and *virG* coding the products VirA and VirG are of the importance in activating the transcription of the other *vir* genes.

The *chv* loci (*chvA, chvB* and *chvE*) play important roles in attachment of the bacteria to plant cell (Cangelosi *et al*., 1987). The *chvA* and *chvB* loci involved in the synthesis and excretion of β - 1, 2 glucan that acts as adhesive or signaling molecules in the attachment of bacteria to the plant cells

(Cangelosi *et al.*, 1989). Meanwhile, *chvE* showed its functional role in bacterial chemotaxis and *vir* genes induction (Ankenbauer and Nester, 1990).

The process of T – DNA transfer involved several essential steps: (1). Bacteria colonisation; (2). *vir* genes induction; (3). T – DNA complex transfer; (4). T – DNA integration (de la Riva *et al.*, 1998) as illustrated in **Figure 2.3**.

Bacterial colonisation takes place when the *Agrobacterium* attached on the plant cell surface with the aids of polysaccharide on the *Agrobacterium* cell surface (Bradley *et al.*, 1997). This polysaccharide appears to be the products of the *Agrobacterium* chromosomal 20 kb *att* locus (Thomashow et al., 1987).

When the *Agrobacterium* perceives signals such as phenolics and sugars being released by the wounded plant cells, the *vir* genes operons (*virB*, *virC*, *virD*, and *virE*) are co-ordinately activated by VirA-VirG components when VirA autophophorylated itself and further phophorylate virG product (Galun and Breiman, 1998). The activation of *vir* genes operons generates single-stranded (ss) molecules of the bottom strand of T–DNA by nicking upon recognition of the T–DNA LB and RB borders by the proteins VirD1 and VirD2 (Zupan and Zambryski, 1995). VirD2 protein remains covalently attached to 5'–end of the ss T – DNA and protects it from exonucleolytic

degradation and distinguishes them as the leading end of T – DNA transfer complex (Dürrenberger *et al.*, 1989).

The ss T – DNA – *vir* D2 complex is exported from the bacterial cell by a 'T-pilus' composed of proteins encoded by the *virB* operon and *virD4* (Dandekar and Fisk, 2005). In the meantime, VirC1 protein repairs and synthesis the displaced strand (Scheppler *et al*., 2000). Once inside the plant cytoplasm, the virE2 proteins cover the ss T – DNA, facilitates nuclear localization and leads T – DNA – VirD2 complex to passage through the nuclear pore complex (NPC) in correct confirmation (Citovsky *et al*., 1992; Zupan *et al.*, 1995). The nuclear localization signal (NLS) of VirD2 and VirE2 direct the T – DNA towards plant cell chromatin (Bravo Angel et al., 1998) and promote integration by illegitimate recombination (Gheysen et al., 1991). Once integrated, repair mechanism of the plant cell will be activated for its own DNA (Puchta, 1998).

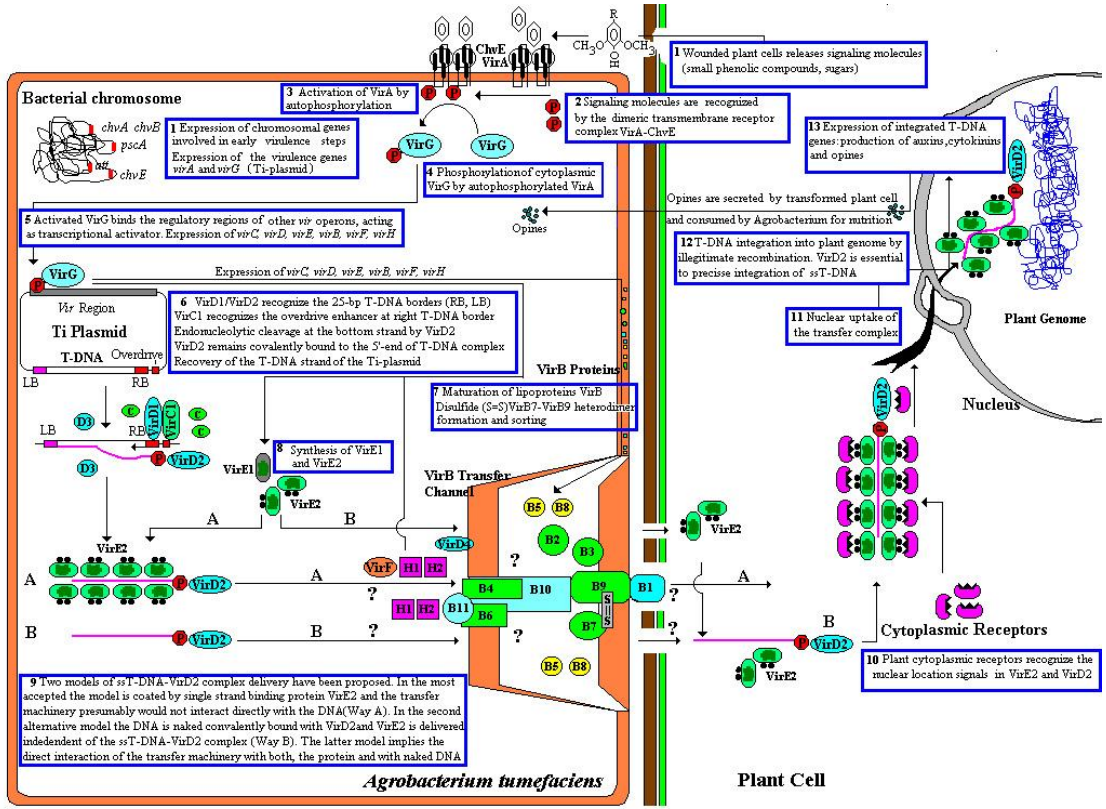

Figure 2.3: *Agrobacterium*- mediated gene transferring mechanisms. With every steps described in the text in boxes

Source: de la Riva *et al.*, 1998

2.3.1.6 Vectors for *Agrobacterium* – mediated transformation

The progression of *Agrobacterium* – mediated transformation is associated with the modification in the Ti plasmid. Non-essential regions of the plasmids are removed including the genes for auxin and cytokinin synthesis were also removed to prevent tumour formation on the transformed plants (Potrykus *et al*., 1998). Disarmed plasmid was reported to prevent recalcitrant attempts of regeneration into whole plant (Zambryski *et al.*, 1983). Besides, unique restriction sites were introduced for inserting foreign genes and recombinant plasmid construction. Additional selectable markers, reporter genes and desirable plant promoters were included inside the LB and RB borders of T – DNA (Gruber and Crosby, 1993). Basically, two main types of vectors have been developed for *Agrobacterium* – mediated transformation in plants – The binary vectors and co - integrate vectors.

2.3.1.6.1 Binary vectors

Binary vectors strategy involved a two plasmid system with the T – DNA and the transfer machinery (the *vir* genes) positioned on separate plasmid. The plasmid carrying the T – DNA regions that will be transferred to plants are thus termed “binary vectors” and the plasmid carrying the vir genes are thus termed “helper plasmid” or “virulence plasmid” (Hoekema *et al.*, 1983). This

strategy is based on the basis that the *vir* genes are able to functions in *cis* orientation. Both vectors are simultaneously involved in transformation, either the binary or the virulence plasmid is not able to transform a plant cell on its own.

Since the first binary vector pBIN19 being constructed by Bevan (1984), many modifications have been made to expand their utility and efficiency. Numbers of new binary vectors with different *Agrobacterium vir* helper strains were developed. A classic binary vector system comprising of an octopine – type *vir* helper strain such as LBA4404 (Hoekema *et al.*, 1983) that harbour the disarmed Ach5 Ti plasmid and a binary vertor such as pBIN 19. Another useful *vir* helper strains is the L, L – succinamopine – type EHA101 (Hood *et al.*, 1986) and EHA105 (Hood *et al.*, 1993) which harbouring the 'supervirulent' *vir* genes which exhibits broader host range and higher efficiency in transformation (Veluthambi *et al.*, 2003).

2.3.1.6.2 The pCAMBIA vectors

The pCAMBIA vector is derivatives of pPZP family of *Agrobacterium* binary vectors (Hajdukiewicz *et al.*, 1994). The vectors offer several advantageous features in which it contained a wide range of unique restriction sites for advance construction, produced high copy number in *E. coli* and stable replication in *Agrobacterium*, convenient bacterial and plant selection marker genes.

pCAMBIA1304 (as shown in **Figure 2.4)** is 12361 bp in size, containing a hygromycin (*hyg*) resistant gene at the LB of transferred region (Hajdukiewicz *et al.*, 1994). Since the RB is a leading first in T–DNA transfer process, hygromycin resistance is present only when the passenger gene is obtained by the plant cell. Besides, it possesses *mgfp5:gusA* fusion genes as the reporters and kanamycin resistant for bacterial selection.

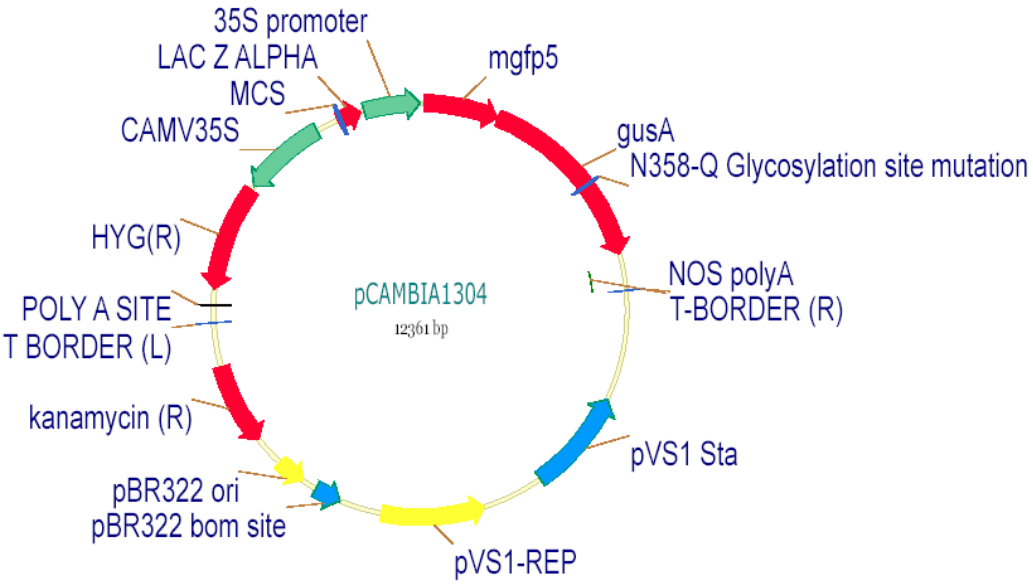

Figure 2.4: The pCAMBIA1304 vector

Source: www.cambia.com

2.3.2 Genetic transformation in aquatic plants

Genetic transformation of the aquatic plant was first reported in the duckweed *Lemna gibba* and *Lemna minor* via *Agrobacterium*–mediated gene transfer method (Yamamoto *et al.*, 2001). These small species of aquatic plants were exploited as ideal plants for bioremediation and large scale production of important recombinant proteins and biomass due to ease of propagation, fast growth rate and high protein yields (Gasdaska *et al.*, 2003). With the optimized glycosylation through RNA interference (RNAi) construct, human monoclonal antibodies (mAb) against CD30 used in the treatment of Hodgkin lymphoma and anaplastic large cell lymphoma were produced in the aquatic plant *Lemna minor* (Cox *et al.*, 2006). Endogenous glycosylation *in L. minor* was silenced by the expression a single RNAi transcript which further allowed The development of *Lemna* Expression System (LEX System) which provides a robust and well–controlled method for clonal propagation of transgenic *L. minor* and recombinant mAb production.

2.4 The reporter systems

Reporter genes are crucial elements in plant transformation vectors, as a means of assessing gene expression and as easily scored indicators of transformation. Sometimes, they are used in place of selectable markers (Slater, 2003). Besides, they are useful tools for the study and analysis of regulatory elements (Thomas, *et al.*, 1990). Examples and the origin of several important reporters are summarised in **Table 2.2**. Amongst all, only a small number of the reporter gene are in widespread use, these being β – glucuronidase (*uidA* or *gus*), green fluorescent protein (*gfp*), luciferase genes (*lux* and *luc*), and the chloramphenicol acethyltransferase gene (*cat*).

Reporter genes are important for establishment of optimal conditions for transformation. Particularly in the case of *Agrobacterium*–mediated transformation wherein complex processes are involved and many aspects of the mechanisms still remain unknown (de la Riva *et al.*, 1998).

Reporter genes	**Abbreviation**	**Source of gene**	**Detection methods**
β – glucuronidase (Jefferson *et al.*, 1987)	*uidA / gus*	*E. coli*	Fluorometric assay; Histochemical staining
Green fluorescent protein (Haseloff *et al.*, 1997)	*Gfp*	*Aequorea victoria* (jellyfish)	Fluorescence
Luciferase (Ow *et al.*, 1986)	*Luc*	*Photinus pyralis (firefly)*	Luminescence
Luciferase (Koncz et al., 1987)	*luxA, luxB*	*Vibrio harveyi*	Luminescence
Chloramphenicol acethyltransferase gene	*Cat*	*E. coli*	Radioactive assay

Table 2.2: Examples and origin of several important reporter genes used in plant transformation

Source: Slater *et al.*, 2003

2.4.1 The β – glucuronidase reporter gene

β – glucuronidase gene appears to be the most widely used reporter genes in plant transformation vectors. The product of this gene (GUS) is a hydrolase that catalyses the cleavage of a variety of β – glucoronides. It can be assayed easily, quickly without involving radioactive methods (Jefferson *et al.*, 1987). Quantitative data can be obtained utilising fluorogenic substrates such as 4 – methylumbelliferry-β-D-glucuronide (4–MUG). Meanwhile, the chromogenic substrate 5-bromo-4-chloro-3-β-D-glucuronide (X–gluc) is used in histochemical staining assay to obtain qualitative results. Besides, it has an advantage because there is little or no GUS endogenous activity in most plant cells.

2.4.2 The green fluorescent protein

The green fluorescent protein (GFP) originally isolated from the bioluminescent jellyfish *Aequorea victoria* emits bright green light that is proportional to the amount of protein present upon excitation of long - wavelength ultraviolet (uv) or blue light (Morise *et al.*, 1974). Its intrinsic, cell-autonomous fluorophore formed autocatalytically without any requirement or substance except for oxygen (Cody *et al.*, 1993). It finds immense applications in every field of biological sciences, especially in

genetic engineering of plants. It allows direct visualisation of gene expression in living cells without the need for invasive methods and addition of toxic substrates. Thus, it serves as a continuous "real–time" screenable marker for transgene expression in transgenic plant cells (Chalfie *et al.*, 1994). It has been widely used as a non-destructive reporter system for both monocots and dicots (Elliot *et al.*, 1998, 1999).

Niedz *et al.* (1995) reported the first transgenic plant with inserted jellyfish *gfp* gene. The group demonstrated successful expression of GFP protein in *Citrus sinensis* protoplasts. Though, some reported poor or no fluorescence in *Arabidopsis* cells and plants transformed with wild type *gfp* gene (Haseloff and Amos, 1995; Hu and Cheng, 1995; Sheen *et al.*, 1995). This setback has been prevailed over with the detection of an aberrant mRNA splicing of *gfp* gene in *Arabidopsis*. Cryptic intron was then removed by altering the codon usage of *gfp* gene using oligonucleotides-directed mutagenesis to avoid mis-splicing in *Arabidopsis* plants. Bright fluorescence was then restored in *Arabidopsis* plant with proper expression. This modified gene, *mgfp4* was then fused to endoplasm reticulum (ER) targeting peptides to circumvent difficulty in regenerating fertile transgenic *Arabidopsis* plants. Subcellular localisation of the GFP protein had solved the problem wherein accumulation of free radicals generated upon excitation in cytoplasm was toxic to plant cells (Haseloff *et al.*, 1997). Soon, subcellular localisation of

GFP proteins was found to be useful as a marker or tracer for studying recombinant proteins compartmentation *in vivo* (Rizzuto *et al.,* 1995) as well as native proteins transportation along secretory pathway (Kaether *et al.*, 1995).

To meet the demand in getting better reporter gene for plant transformation, more variants or mutant of *gfp* were developed. These variants served the purpose better with enhanced, brighter fluorescence (Davis and Vierstra, 1998; Reichel *et al.*, 1996), increased solubility in cytoplasm (Davis and Vierstra, 1998), better temperature stability (Siemering et al., 1996), shifted excitation and emission spectral (Kato *et al.,* 2002). Most of these improved versions of GFP variants were generated using site-directed mutagenesis methods. Other than this, new fluorescent proteins isolated from different species were also exploited in plant transformation experiments. This included the red fluorescent protein (DsRed) from tropical corals (Clontech Laboratories, California) which was used in *Agrobacterium*-mediated transformation of tobacco mesophyll cells (Kato *et al.*, 2002).

2.5 Molecular assessment of transformant

Dominant selectable markers enable the transformed plant cells to survive and grow under selective conditions that could restrict the growth of wild type plant cells. Most of these genes conferred resistance to antibiotics or herbicides. Several dominant selectable markers that were vastly in use are as summarised in **Table 2.3**. Employment of the selectable markers facilitates elimination of non-transformed plant samples for further examination.

Putative transformed plant samples that have survived in the presence of selective agents and showing positive result in reporter gene assay are subjected to further verification using molecular approaches i.e. Polymerase Chain Reaction (PCR), Southern Blotting, Northern Blotting, Western Blotting, Real-Time PCR and also immunoassay. Verification of putative transformed plant cells with PCR aims to prove the presence of the gene inserted while copy number and integration of the gene inserted was revealed via Southern Blotting analysis. Segregation analysis of transformed plant samples can further shows stable integration of transgene.

Selectable markers	**Abbreviation**	**Source of gene**	**Principle of selection**
Neomycin phosphotransferase	*nptII*	*E. coli*	Antibiotics resistance
Hygromycin phosphotransferase	*hyg*	*Klebsiella spp.*	Antibiotics resistance
Phosphinothricin acethyltransferase	*Bar and pat*	*Streptomyces hygroscopicus*	Herbicides resistance
Glycopeptides-binding protein	*ble*	*Streptalloteichus hindustantus*	Antibiotics resistance
Acetolactate synthase	*Csr1 – 1*	*Arabidopsis thaliana*	Herbicides resistance
Phosphomannose isomerase gene	*pmi*	*E. coli*	Positive selection
Xylose isomerase	*xylA*	*Thermoanaerobacterium thermosulfurogenes*	Positive selection

Table 2.3: Selectable markers and their respective source and principle of selection

Source: Twyman *et al.*, 2002; Veluthambi *et al.*, 2003

2.5.1 Polymerase Chain Reaction (PCR)

PCR is used to verify the presence of transgene in transgenic plants. It is the method developed by Mullis (1983) whereby a region of DNA flanked by short oligonucleotides which act as primers is amplified exponentially by thermostable enzyme polymerase of *Thermus aquaticus* (*Taq* polymerase). *Taq* polymerase is employed in the reaction because denaturation of DNA molecules is required at high temperature for the primers to hybridize before subsequent DNA synthesis is carried out. The cycle of denaturation-hybridization-synthesis is repeated in a thermalcycler machine for 25 – 30 times. Hundred millions of copies can be obtained from trace template of DNA from the exponential amplification.

2.5.2 Southern Blotting

Southern Transfer techniques developed by Southern (1975) whereby DNA fragments separated on electrophoresis gel were transferred onto nitrocellulose membrane via capillary action of high salt buffer. Chromogenic or chemiluminescent reagents often employed to label specific probes used in the detection. Probes will bind to single-stranded DNA on the membrane which has been denatured prior to the transfer. Only complementary sequences will

bind to probes and give out signal after high stringency wash with low salt buffer.

Other than Southern Blot analysis, quantitative real-time PCR was also used in the detection of transgene copy number in transgenic plant studies (Mason *et al*., 2002). However, expensive equipments and skillful personnel are needed. Furthermore, endogenous genome signal is a pre-requisite for the use of Real-Time PCR for the detection of transgene copy number in plant for instance, the signal of endogenous house-keeping gene copy number. Yi *et al.* (2008) used a Taqman quantitative Real-Time PCR detection and validated a single copy of endogenous GhUBC1 gene per haploid cotton genome to estimate copy number of GFP gene and selectable kanamycin gene (*nptII*) number in transgenic cotton. Extensive calibration is also required to obtain a reliable and accurate results using Real-Time PCR.