AGROBACTERIUM–MEDIATED TRANSFORMATION OF *DENDROBIUM* BROGA GIANT ORCHID WITH CHITINASE GENE

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By

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LIST OF ACRONYMS AND ABBREVIATION

ACRONYMS	ABBREVIATION
AA	: Acetic acid
AS	: Acetosyringone
A_{415}	: Light absorption at 415 nm
BAP	: 6-Benzylaminopurine
Bar	: Bialaphos resistance gene
Bp	: Basepairs
CaOx	: Calcium oxide
CM	: Chemotaxis medium
Cm	: Centimeter
cm^3	: Cubic centimeter
DBG	: *Dendrobium* Broga Giant
DNA	: Deoxyribonucleic acid
dTNP	: Deoxyribonucleotide triphosphate
EDTA	: Ethylenediaminetetraacetic acid
EPS	: Exopolysacharide
FAA	: Formalin-acetic-acid-alcohol
G	: Gram
g/L	: Gram per litre
Glu	: Glucose
GM	: Genetically modified
GMO	: Genetically modified organism
GUS	: β-glucuronidase marker
H	: Hour
Kbp	: Kilobasepairs

Kpa	: Kilopascal
LB	: Luria Bertani
M	: Molarity
MIC	: Minimal inhibitory concentration
mg/L	: Milligram per litre
mg/ml	: Milligram per millilitre
$MgCl_2$	: Magnesium Chloride
Ml	: Millilitre
mM	: Millimolar
Mm	: Millimeter
m^{-2}	: Per square meter
MS	: Murashige and Skoog
NAA	: Naphthaleneacetic acid
Nm	: Nanometre
nptII	: Neomycin phosphotransferase II gene
OD	: Optimal Density
PCR	: Polymerase Chain Reaction
PGR	: Plant growth regulator
pH	: Potential hydrogen
Pmol	: Picomole
Rpm	: Rotation per minute
s^{-1}	: Per second
SEM	: Scanning electron microscope
Sp	: Species
TBA	: Tertiary butyl alcohol
TBE	: Tri/Borate/EDTA Buffer

T-DNA	: Transfer DNA
TEM	: Transmission electron microscope
Tm	: Melting temperature
TRIS-HCL	:Tris(hydroxymethyl)aminomethane hydrochloride
UPP	: Unipolar polysaccharide
v/v	: Volume over volume
Vir	: Virulance
W	: Watt
w/v	: Weight over volume
wwin-1	: *wheatwin 1* gene
wwin-2	: *wheatwin 2* gene
X-Gluc	:5-bromo-4-chloro-3-indlyl-beta-D-glucuronic acid cyclohexylammonium salt
Ml	: Microlitre
Mm	: Micrometer
µmol	: Micromole

TRANSFORMASI PERANTARAAN-*AGROBAKTERIUM* UNTUK ORKID *DENDROBIUM* BROGA GIANT DENGAN GEN KITINASE

ABSTRAK

Dendrobium Broga Giant (*Dendrobium Bobby Messina* × *Dendrobium superbiens*) ialah orkid hibrid terbaru di Malaysia. Kajian ini telah menghasilkan protokol transformasi perantaraan-*Agrobakterium* menggunakan jasad seperti protokom (JSP) untuk menghasilkan orkid transgenik *Dendrobium* Broga Giant (DBG) dengan gen anti-kulat. Satu teknik mikropropagasi yang mudah dan kompeten telah dihasilkan untuk percambahan JSP dari DBG. Media Murashige dan Skoog (MS) dengan setengah kekuatana separa pepejal ditambah dengan 1.0 mg/L BAP dan 0.5 mg/ L NAA menghasilkan percambahan JSP tertinggi. Interaksi antara *Agrobakterium* dan JSP dinilai berdasarkan pergerakan bakteria dan keupayaan pelekatan. JSP yang separa luka memaparkan pergerakan *Agrobacterium* serta kapasiti pelekatan yang lebih tinggi berbanding dengan rawatan lain. JSP yang berjaya ditranformasi dinilai dengan menggunakan ejen pemilihan seperti geneticin (G-418), kanamycin dan neomycin untuk menentukan kepekatan perencatan minimum. Geneticin menunjukkan keberkesanan tertinggi dengan 50 mg/L membunuh sepenuhnya JSP yang tidak ditranformasi dan membenarkan kemandirian JSP yang berjaya ditransformasikan. Beberapa faktor (saiz JSP, kelukaan, masa rendaman, tempoh pengkulturan, kepadatan *Agrobakterium*, acetosyringone, L-cysteine dan kepekatan nitrat perak) telah dinilai melalui pengekspresan sementara gen *gusA* untuk mengoptimum protokol transformasi untuk DBG orkid. Berdasarkan keputusan pengekspresan sementara gen *gusA*, saiz 3-4mm JSP separa luka yang direndam selama 15 minit dalam perendaman *Agrobakterium* (OD_{600nm} 0.8) dan pengkulturan bersama untuk 2 hari dalam keadaan gelap di media yang mengandungi 200µM acetosyringone, 60µM nitrat perak dan 200 mg/L L-cysteine telah menghasilkan pengekspresan tertinggi gen *gusA* dalam DBG orkid. Akhir sekali, gen kitinase *wwin1* dan *wwin2* telah dimasukkan ke JSP DBG melalui sistem transformasi pengantaraan-*Agrobakterium* untuk menghasilkan orkid DBG transgenik yang rintang kulat. Bilangan transforman yang putatif dengan gen *wwin1* dan *wwin2* telah menghasilkan 18.13% dan 17.60% selepas satu bulan di bawah pemilihan geneticin. Pengesahan terakhir tumbuhan transgenik telah disahkan oleh tindak balas rantai polymerase (PCR) dan analisis polimorfik ditentukan oleh penanda molekul DAMD. Tahap polimorfik maksimum (13.51%) telah diperolehi dalam *wwin1* yang mempunyai tumbuhan transgenik baris 4 dan *wwin2* pokok transgenik baris 8. Walau bagaimanapun, tahap polimorfik minimum (5.41%) direkodkan daripada gen *wwin1* yang mempunyai pokok transgenik baris 1. Analisis biokimia seperti jumlah klorofil, karotenoid, porphyrin, karbohidrat dan proline telah ditentukan dalam setiap tumbuhan transgenik dan menunjukkan kandungan yang lebih tinggi daripada anak tumbuhan kawalan yang tidak ditransformasi. Oleh itu, kejayaan transformasi JSP DBG mendedahkan bahawa semua tumbuhan transgenik adalah dianggap tahan kulat dan ia juga berkemungkinan untuk memperkenalkan gen lain bagi penambahbaikan ciri agronomi hibrid orkid ini.

AGROBACTERIUM–MEDIATED TRANSFORMATION OF DENDROBIUM BROGA GIANT ORCHID WITH CHITINASE GENE

ABSTRACT

Dendrobium Broga Giant (*Dendrobium Bobby Messina* × *Dendrobium superbiens*) is a new hybrid orchid in Malaysia. This study has established *Agrobacterium*-mediated transformation protocol using protocorm-like bodies (PLBs) to produce transgenic *Dendrobium* Broga Giant (DBG) orchid with an antifungal gene. A simple and competent micropropagation technique was established for the successful PLBs proliferation of DBG. Half-strength of Murashige and Skoog (MS) semi-solid media supplemented with 1.0 mg/L BAP and 0.5 mg/L NAA produced the highest PLBs proliferation. The interaction between *Agrobacterium* and PLBs was evaluated based on bacterium motility and attachment capability. Mildly wounded PLBs displayed higher *Agobacterium* motility as well as attachment capacity compared to other treatments. Successfully transformed PLBs were evaluated using selection agents such as geneticin (G-418), kanamycin and neomycin to determine the minimal inhibitory concentration required. Geneticin displayed highest effectiveness with 50 mg/L completely killed the non-transformed PLBs and allowed the survival of the transformed PLBs. Several factors (PLB size, wounding, immersion time, co-cultivation period, *Agrobacterium* density, acetosyringone, L-cysteine and silver nitrate concentrations) were evaluated via transient *gusA* gene expression for the optimisation of transformation protocol for DBG orchid. Based on the transient *gusA* gene expression results, 3-4mm size mildly wounded PLBs immersed for 15 minutes in the *Agrobacterium* suspension ($OD_{600\ nm}$ 0.8) and co-cultivated for 2 days in dark condition on MS medium containing 200μM acetosyringone, 60μM silver nitrate and

200 mg/L L-cysteine produced highest *gusA* gene expression in DBG orchid. Finally, chitinase genes *wwin1* and *wwin2* were introduced to DBG's PLBs via *Agrobacterium*-mediated transformation system to produce fungal resistance transgenic DBG orchid. Number of putative transformants with *wwin1* and *wwin2* genes was obtained at 18.13% and 17.60% after one month under geneticin selection. Final confirmation of transgenic lines was confirmed by polymerase chain reaction (PCR) and polymorphic analyses were determined by DAMD molecular marker. The maximum polymorphic level (13.51%) was obtained in *wwin1* carrying transgenic line 4 and *wwin2* carrying transgenic line 8. However, the minimum polymorphic level (5.41%) was recorded from the *wwin1* gene carrying transgenic line 1. Biochemical analyses such as total chlorophyll, carotenoid, porphyrin, carbohydrate and proline were determined in each transgenic line and shown higher contents than the non-transformed control plantlets. Hence, the successful transformation of DBG's PLBs revealed that all transgenic lines are considered potentially fungal resistant and it is also possible to introduce other genes for improvement of agronomic traits in this orchid hybrid.

CHAPTER 1

GENERAL INTRODUCTION

Orchids are important ornamentals and have became the second largest cut flowers and potted floricultural crop (Hossain et al., 2010). Orchids occupy the leading position in floriculture industry particularly in ornamental flower business due to its attractive colours, ability to produce flowers continuously and a prolonged vase life in comparison to other species (Kuehnle, 2007; Sugapriya et al., 2012; Teixeira da Silva et al., 2014). *Dendrobium* is one of the largest genuses under orchidaceae family which represents more than 1,100 species around the world (Luo et al., 2008; Adams, 2011). *Dendrobium* species are either epiphytic or occasionally lithophytic and is commonly abbreviated as 'Den' in horticulture science. *Dendrobium* Broga Giant is a new orchid hybrid which was crossed between *Dendrobium Bobby Messina* and *Dendrobium superbiens* hybrids.

In general, orchids represents second position under potted flowering plants in the United States and earned US$ 200 million for the year 2011 (NASS 2013). However, the USDA floriculture report of 2013 indicated an 11% increased in the value of potted orchids in Year 2012 compared to the previous year (NASS 2013). Thailand earned USD 80 million from orchid exportation in 2009 (Supnithi et al., 2011) and Taiwan earned US$82.55 million in 2010 (Australia- Taiwan Orchid media release, 2011). Malaysia exports orchids as cut-flower like other Asian counterparts such as Thailand and Philippines. In Malaysia, RM 150 million is annually generated from orchid exportation which represents approximately 40% of the total floriculture product exportation (Ahmad et al., 2010).

Orchids grown in Malaysia and Thailand are highly susceptible to pathogen attack due to the warm and humid climate with high night temperature. Diseases caused by bacterial (eg. *Erwinia corotovora*), fungal (*Fusarium proliferatum*), and viral (*Cymbidium* Mosaic Virus: CymMV) infection causes major loss of yield with detectable effects such as flower and leaf discolouration, stunted growth and death of young plant (Chang et al., 2005; Sjahril et al., 2006). *Dendrobium* orchid is susceptible to most of the fungal diseases especially to *Fusarium proliferatum* race B which caused black spot disease on *Dendrobium's* leaf resulting in adverse effect on orchids and stunted its growth (Ichikawa and Aoki, 2000).

Many orchid farmers are still lagging in gratifying the international orchid demand, as the main rationale is that the orchids are still being produced via conventional breeding methods. *Dendrobium* is susceptible to most of the fungal diseases, mostly in the humid climate area (Ichikawa and Aoki, 2000). The development of fungal resistance *Dendrobium* orchid via traditional breeding methods with sexual hybridization and selection of polyploids with variants is inadequate due to time consumption and difficulties in obtaining the desired traits. Conventional breeding techniques involved traditional hybridization to transmit valuable traits into commercial varieties and followed by exposure of novel varieties is laborious and time consuming method that takes about 2 – 13 years (Sim et al., 2007; Tang and Chen, 2007). Modern biotechnological approaches such as micropropagation and genetic engineering offer a promising alternative breeding platform to solve such problems.

Micropropagation is the most important and widely accepted convenient application in plant biotechnology that has gained the status of a multibillion-dollar industry all over the world (Winkelmann et al., 2006; Liao et al., 2011). Micropropagation is asexual propagation practices which rapidly multiply orchid plantlets from any orchid cells, tissue or organs by using modern tissue culture techniques (as reviewed by Arditti, 2008). Micropropagation is comparatively easy to perform and shortens the orchid's life cycle during the modification of nutrient rich medium and the establishment of similar microhabitat for the orchid to grow vigourously.

Genetic engineering is the process of manually introduction of foreign DNA to an organism (Jauhar, 2006). Genetic engineering also known as genetic transformation, involve physically removing a specific gene from one organism to insert it into another organism which is performed to express the trait encoded by that gene (Yu and Xu, 2007). The introduction of novel features such as disease resistance, drought tolerant, salinity tolerant or new colour into orchid is generally not easy during mutation or conventional breeding. However, this could be achieved comparatively easily through genetic engineering (Nirmala et al., 2006; Mii and Chin, 2010). The most popular techniques to create transgenic orchids through genetic engineering are *Agrobacterium*–mediated transformation, microparticle bombardment (biolistics) and electroporation (as reviewed by Hossain et al., 2013). *Agrobacterium*-mediated transformation is the most commonly used techniques over biolistics and electroporation because it can introduce larger segments of DNA with minimal relocation and with fewer number of inserted transgenes at higher efficiencies (Shou et al., 2004; Shewry et al., 2008; Pitzschke and Hirt, 2010; He et al., 2010). Monocots including orchids are not the natural host of *Agrobacterium*. Hence, the initial trials were complicated on the transformation of orchids by *Agrobacterium*-mediated technique (as reviewed by Teixeira da Silva et al., 2011). To overcome the complexity, several factors such as *Agrobacterium* strain, density, selection agent, plasmid construct, wounding type, immersion time, co-cultivation period, acetosyringone, silver nitrate, L-cysteine and source of explants have been taken into consideration.

Selection of transformants is a critical stage in developing a transgenic plant. The most convenient approach for transgene detection is using insertion of a marker gene that offers positive or negative selection of transformants or a gene that provides scorable visual phenotypes (Pinkerton et al., 2012). The use of suitable selection marker plays an important role in the improvement of transformation method (Opabode, 2006). Selectable marker gene is used for selecting the transformants and to exclude nontransformants explants. Selective agents are toxic to non-transformed cells above a certain concentration. However, transformed cells will survive when cultured in the presence of that chemicals (Yu and Xu, 2007; Hsiao et al., 2011). Commonly used selection markers genes in orchid transformations are neomycin phosphotransferase (*nptII*), hygromycin phosphotransferase (*hpt*), and phosphinothricin-N acetyl transferase (*bar*) presenting resistant to kanamycin, hygromycin and phosphinithricin respectively (Jardak-Jamoussi et al., 2008). Gene *nptII* coding used for neomycin phosphotransferase detoxifies aminoglycoside compounds like kanamycin, neomycin and geneticin (Yong et al., 2006a).

Generally, expression of a gene is synchronized by relations between its promoter and the transcription factors allocating the gene to act in response towards various stimuli as well as tissue localization and environmental factors (Rosellini, 2011). Promoters are assessed by determining the expression of reporter genes such as β-glucuronidase (*gusA*) (Gnasekaran et al., 2014b), green fluorescent protein (*gfp*) (Thiruvengadam et al., 2011a), and luciferase (Bourdon et al., 2002) to enumerate a novel gene expression. Gene *gusA* is the most frequently used reporter gene to detect the transformation events (Jefferson et al., 1987). The expression of *gusA* gene is simply visualized during the activity of *de novo* synthesized β-glucuronidase (*gusA*), which provides the blue colour of transformed cells through catalyzing the exogenously applied substrate 5-bromo-4-chloroindoyglucuronidase (X-gluc).

Microorganisms such as bacteria, fungi, protozoa, nematode, parasite and viruses cause infectious diseases to plant (Singh et al., 2011). Ectoparasites like insects, mites and vertebrates also affect plant health by initiating pathogenic attack. Hence, plants employ defense approach to prevent the assault of pathogens and to struggle against disease caused by microorganisms via synthesis of pathogenesis related (PR) proteins (Kim et al., 2014). PR proteins are recognized to function in higher plants in opposition to abiotic and biotic stresses mainly against pathogen infection (Pereira Menezes et al., 2014).

The proposed research study involves transfer of antifungal chitinase genes into *Dendrobium* Broga Giant orchid's protocorm-like bodies (PLBs) with *nptII* as a selectable marker gene for the production of fungal disease-resistant plantlets. *Dendrobium* Broga Giant orchid will be transformed into PLBs using *Agrobacterium tumefaciens* strain LBA 4404 harbouring vectors (pW2KY and pW1B1). Both pW2KY and pW1B1 plasmids contained antibiotic resistance and chitinase genes

1.1 Objectives

The main objectives of the present research are:

i. To optimise *in vitro Dendrobium* Broga Giant orchid PLBs proliferation,

ii. To determine chemotaxis movement and attachment of *Agrobacterium* on *Dendrobium* Broga Giant orchid's PLBs,

iii. To identify suitable novel encapsulation-based antibiotic selection technique,

iv. To establish an *Agrobacterium*-mediated transformation system using the transient *gusA* reporter gene for optimization purpose,

v. To confirm a successful production of transgenic *Dendrobium* Broga Giant orchid using PCR and polymorphism analysis through DAMD molecular marker,

vi. To determine total chlorophyll, carotenoid, porphyrin, carbohydrate and proline contents in each transgenic plantlets.

CHAPTER 2

LITERATURE REVIEW

2.1 Orchid

Orchids are monocotyledonous plants under *orchidaceae* family which includes five sub families such as *Apostasiodae, Cypripoideae, Epidendroideae, Orchidoideae* and *Vanilliodeae* (Cameron, 2001; Chase et al., 2003). Orchids are important members of the botanical world because of their morphological, physiological and ecological specializations (Fay, 2010). *Orchidaceae* is the largest family in angiosperms which covers about 34 % of monocots plant (Soltis and Soltis, 2004) and 7-10% of all flowering plants species (Benner et al., 1995; Hagsater and Dumont, 1996).

Orchid is an important group of ornamental plants comprising of 800 genera and 25000 species with most of them grown in tropical regions (Hamdan, 2008; Chugh et al., 2009; Nambiar et al., 2012). More than 120 genera and 2000 species of orchid were discovered in Malaysia (Hamdan, 2008). Chugh et al. (2009) reported that million dollar orchid industries are generated from Thailand, Australia, Singapore and Malaysia which are covers 8% of the world floriculture trade.

2.1.1 The genus *Dendrobium*

Dendrobium is the second largest orchid genus in the world after *Bulbophyllum* (Puchooa, 2004) which has more than 1200 *Dendrobium* species around the world and is extremely valued in the ornamental flower industries (Luo et al., 2008; Adams, 2011). *Dendrobium*s are a perennial herb, sympodial and mostly are epiphytes orchids (Kuehnle, 2007). *Dendrobium* is the most popular orchid and commercially grown in tropical countries such as Malaysia and Thailand (Akter et al., 2007).

Dendrobium hybrids are mainstream orchids in floriculture trade especially in ornamental flower industry because of its elegant colours, continuous flowering ability and a prolonged self life when compared with other orchid species (Nambiar et al., 2012). *Dendrobium* is used as cut flowers or potted plants for landscaping purposes because of their high potentiality (Sugapriya et al., 2012). Malaysia earns RM 150 million annually from orchid exportation where *Dendrobium* individually contributed 11.7% of total exportation (Ahmad et al., 2010).

Dendrobium is also used in cosmetic and medicinal industries. Shiau et al. (2005) reported that sesquiterpene alkaloids derived from *Dendrobium* are used in Chinese traditional medicine as tonic for improving digestion, increase body-fluid production and reducing heat (Shiau et al., 2005). The stem of *Dendrobium huoshanense* is used against stomach and ophthalmic disorder (Hsieh et al., 2008). Singh et al. (2007) reported that *Dendrobium* was used as an Ayurvedic indigenous medicine in India. Different *Dendrobium* species such as *Dendrobium* 'Second Love' (Ferreira, 2003; Campos and Kerbauy, 2004; Ferreira et al., 2006), *Dendrobium* 'Chao Praya Smile' (Hee et al., 2007), *Dendrobium* 'Madame Thong-In' (Sim et al., 2007; 2008), *Dendrobium* sonia-17 (Tee et al., 2008), *Dendrobium* sonia-28 (Julkifle et al., 2014), *Dendrobium denndanum* (Guan and Shi, 2009), Friederick's *Dendrobium* (Te-chato et al., 2009), *Dendrobium nobile* Lindl (Wang et al., 2009), *Dendrobium primulinum* (Deb and Sungkumlong, 2009), *Dendrobium officinale* (Cen et al., 2010), *Dendrobium strongylanthum* (Zhao et al., 2012), *Dendrobium aphyllum* (Hossain et al., 2013), *Dendrobium* 'Chao Praya Smile', 'Pinky' and 'Kiyomi Beauty' (Ding et al., 2013), *Dendrobium wangliangii* (Zhao et al., 2013) and *Dendrobium officinale* (Wang et al., 2014) were used for micropropagation studies.

2.2 Micropropagation for orchid development

Micropropagation provided a significant breakthrough for mass proliferation of many orchid species which have greatly heterozygous genotype and have extremely slow reproduction ability (Kanjilal et al., 1999). Rout et al. (2006) reported that micropropagation is a powerful tool for large scale propagation within short period of time and the best method for eliminating pest infestation.

An initial step of micropropagation involves the collection of plant materials and surface sterilization to eliminate fungus, bacteria and viruses on the plant materials. Any plant parts (leaf, shoot tips, root tip, leaf mid-rib, cotyledons etc) can be used as starting materials for micropropagation. For example, cotyledons (Dai et al., 2014; Chowdhury et al., 2014), apical meristem (Arthikala et al., 2014), protocorm-like bodies (Zhao et al., 2013; Ding et al., 2013; Julkifle et al., 2014; Gnasekaran et al., 2014; Wang et al., 2014), callus (Ramadevi et al., 2014), leaf midribs (An et al., 2014), leaves (Sheelavanthmath et al., 2005; Jua et al., 2014; Monemi et al., 2014; Slazak et al., 2015), shoot-tip (Tokuhara and Mii, 2001; Roy and Banerjee, 2003; Babaei et al., 2014), embryo (Roly et al., 2014; Delporte et al., 2014; Sood et al., 2014) and anther (Hoseini et al., 2014) were used as starting materials for a successful micropropagation.

For orchid regeneration, target explants are used to generate *in vitro* cultures which are initiated on nutrient rich medium. Dutra et al. (2008) reported that *in vitro* plant materials are chopped into small pieces and transferred to new media for successful regeneration. Orchid producing countries such as Thailand, Taiwan, Malaysia, Japan, and Netherlands are using micropropagation techniques for mass and quality propagation of orchids (Thammasiri, 1997; Griesbach, 2002; Chugh et al., 2009). Micropropagation technique were successfully used in various orchid species such as *Aerides maculosum* (Murthy and Pyati, 2001), *Arachnis*, *Malaxis*, *Cleisostoma* (Deb and Imchen, 2010), *Dendrobium* sp., (Rangsayatorn, 2009; Pornpienpakdee et al., 2010; Julkifle et al., 2014), *Laelia speciosa* (Avila-Diaz et al., 2009), *Oncidium* sp., (Kalimuthu et al., 2007) and *Vanda* (Kishor and Devi, 2009; Gnasekaran et al., 2014) for regeneration purposes.

2.3 Micropropagation media

Different types of media such as Hyponex medium (Kano, 1965), Murashige and Skoog (1962), Knudson C (1946), Vacin and Went (1949) were used for *in vitro* orchid culture. Murashige and Skoog (MS) media widely used in *Heliotropium kotschyi* (Sadeq et al., 2014), Japonica rice (Sah et al., 2014), black cardamom (Pradhan et al., 2014), *Aerva lanata* (Varutharaju et al., 2014), strawberry (Kadhimi et al., 2014), and *Ornithogalum* (Lipsky et al., 2014) for successful micropropagation. The modification micropropagation media by addition of organic substrate, plant growth regulators (PGRs) and carbon sources are essential to improve orchid growth (Ferreira et al., 2006; Thomas, 2008; Avila-Diaz et al., 2009; Rahman et al., 2009). Gnasekaran et al. (2014b) highlighted that Vacin and Went medium supplemented with coconut water and tomato extract were suitable for *Vanda* Kasem's Delight Tom Boykin (VKD) orchid *in vitro* propagation.

Furthermore, proliferation of certain orchid species such as *Dendrobium* and *Vandofinetia* via protocorm-like bodies (PLBs) produced significant growth rate in MS medium compared to Knudson C solution, Vacin and Went medium and new *Phalaenopsis* medium (Kishi et al., 1997; Akter et al., 2008). MS media were generally used for the *in vitro* propagation of *Aerides* (Murthy and Pyati, 2001), *Anoectochilus elatus* (Sherif et al., 2014), *Cymbidium* (Gao et al., 2014; Ghosh et al., 2014), *Dendrobium* (Martin and Madassery, 2006; Akter et al., 2008; Julkifle et al., 2014; Chen et al., 2014; Habiba et al., 2014), *Gramatophyllum* (Pimsen and Kanchanapoom, 2011), *Orchis catasetum* (Baker et al., 2014 ; Ghaziani et al., 2014), *Phalaenopsis* (Sinha et al., 2010) and *Pogostemon cablin* (Swamy et al., 2014).

2.3.1 Liquid and semi-solid media culture

There are two types of micropropagation media in culture system such as semi-solid or liquid culture media. The liquid media culture system provides a simple process for plantlets immunization and requires continuous shaking on automated rotator or roller type bioreactors (Park et al., 2000; Wawrosch, 2010). The constant shaking of the liquid culture provides oxygen to plantlets for appropriate respiration and dispersed nutrient equally to encourage the initiation and explosion of numerous auxiliary buds (Kanjilal et al., 1999; Mehrotra et al., 2007). The MS liquid media are also used in strawberry (Kadhimi et al., 2014) and *Ornithogalum* (Lipsky et al., 2014) for successful micropropagation.

The semi-solid media is solidified by gelling agent like agar, gelrite or gellan gum. The semi-solid media affords surrounding substances to support plants over the surface of the media and in vertical position of plants (Prakash et al., 2004). Ibrahim et al. (2005) found that gelling agent is important for some species to prevent hyperhydricity which is adverse stipulation in plant physiology. Culture system provides significant effects on the *in vitro* proliferation of PLBs of *Phalaenopsis* and *Doritaenopsis* orchids (Liu et al., 2006). Julkifle et al. (2014) highlighted that the half-strength semi-solid MS media provides highest proliferations rate of *Dendrobium* sonia – 28 orchid’s PLBs. Half-strength of MS media was suitable for *in vitro* propagation of *Brassocattleya* (Cardoso and Ono, 2011) and wild medicinal orchid of *Coelogyne cristata* (Naing et al., 2011).

2.3.2 Carbon sources

Different types of sugars such as sucrose, fructose, maltose, sorbitol and glucose are frequently used as carbon sources for *in vitro* propagation of orchids. Murdad et al. (2010) stated that proper doses of sugars play an important role in orchid micropropagation. Udomdee et al. (2014) confirmed that sucrose provided energy to culture medium and tissues absorb energy easily for their primary growth. Zha et al. (2007) reported that, sucrose could provide a balanced carbon source in medium by glycolytic and pentose phosphate pathways for proper cell growth.

Sucrose supply energy for orchid respiration (Hew and Yong, 2004) and synthesis of protein and lipids in plants (Yu et al., 2001). Deb and Pongener (2011) observed that different concentration of sucrose directly affected the immature seed germination of *Cymbidium aloifolium*. Vijaykumar et al. (2012) also reported that MS medium supplemented with 3% sucrose and coconut water provides higher germination rates, supports PLBs proliferation and maximises the number of shoots in *Dendrobium aggregatum* orchids. Increasing the sucrose concentration from 10 to 50 mM decreased germination percentages as well as development of *Bletia purpurea* (Johnson et al., 2011). In addition, Jawan et al. (2010) found that sucrose is more superior to other sugars for *in vitro* propagation of orchids. Carbon source is also known to play an important role in osmotic potentiality to morphogenesis development of *in vitro* plantlets (Paivo-Neto and Otoni, 2003).

2.3.3 Plant growth regulators

Plant growth regulators (PGRs) are one of the supportive resources of controlling plant organogenesis *in vitro* (Teixeira da Silva, 2014). Different plant growth regulators (PGRs) such a benzylaminopurine (BAP), naphthaleneacetic acid (NAA), thidiazuron (TDZ), kinetin (Kin) and indole-3-acetic acid (IAA) are used for the micropropagation of orchids (Sheelavanthmath et al., 2005; Zhao et al., 2008).

PGRs enhanced micropropagation of different orchid species such as *Cypripedium formosanum* (Lee and Lee, 2003), *Doritis pulcherrima* Lindl (Mondal et al., 2013), *Dendrobium kingianum* (Habiba et al., 2014), *Cymbidium* Twilight Moon 'Day Light' (Fujii et al., 1999, Hamada et al., 2010, Teixeira Da Silva, 2014), *Epidendrum* 'Rouge Star No. 8' (Habiba et al., 2014), *Phalaenopsis amabilis* (Ori et al., 2014), *Phalaenopsis aphrodite* (Feng and Chen, 2014), *Dendrobium huoshanense* (Lee and Chen, 2014), *Dendrobium aggregatum* Roxb.(Hossain, 2013), *Malaxis acuminata* D. Don a (Deb and Arenmongla, 2014), and *Dendrobium* sonia- 28 (Martin and Madassery, 2006; Julkifle et al., 2014; Swamy et al., 2014).

2.4 Protocorm-like bodies (PLBs)

Protocorm-like bodies (PLBs) are somatic embryos which are unique *in vitro* structures that limited to orchids (Tisserat and Jones, 1999) and earliest structure formed during embryo development in orchid seed germination (Arditti, 2008). PLBs are commonly used target explants for the establishment of orchid culture because of their rapid proliferation capacity (Men et al., 2003a).

PLBs are made of actively dividing cells and produce secondary PLBs which is also important for transformation and proliferation of new shoots (Kuehnle and Sugii, 1992; Park et al., 2002; Liau et al., 2003b). Studies conducted by Mishiba et al.

(2005), confirmed that regenerated PLBs are genetically uniform; an essential factor for transformation studies. Yu et al. (2001) reported that *Dendrobium* PLBs generate higher level of phenolic substances such as coniferyl alcohol that acts as signalling molecule. PLBs are the most suitable target explants for genetic transformation of orchids (Belarmino and Mii, 2000; Chai et al., 2002; Suzuki and Nakano 2002; Men et al., 2003b; Mishiba et al., 2005; Ravindra et al., 2007) because of their organized structure and rapid regeneration capacity (Yang et al., 2003; Sreeramanan et al., 2009a).

Successful transformation of different orchid species and hybrids such as *Amitostigma hemipilioides* (Yang et al., 2014), *Cattleya* (Zhang et al., 2010), *Cymbidium* (Chin et al., 2007), *Dendrobium officinate* kimura et migo (Quan et al., 2010), *Dendrobium nobile* Lindl (Bhattacharyya et al., 2014), *Dendrobium* sonia-28 (Julkifle et al., 2014), *Oncidium* (Liu et al., 2014), *Phalaenopsis* (Chai et al., 2002; Samarfad et al., 2014) and *Vanda* hybrids(Shrestha, 2007; Gnasekaran et al., 2014b) have been reported by using PLBs as the target explants.

2.4.1 Microscopic analysis of PLBs through SEM and TEM

Scanning electron microscopy (SEM) analysis focuses on the sample's surface area as well as sample morphology and transmission electron microscopy (TEM) provides the details on the internal composition of samples such as morphology, crystallization, stress and even magnetic domains. In terms of morphology analysis of PLBs, SEM and TEM analyses will be conducted to justify that PLB is a meristematic tissue and contains actively dividing cells that is suitable as a target material for *in vitro* system.

Both SEM and TEM analysis have assisted in the understanding of the formation and the development of PLBs. Aslam et al. (2011) analyzed dense cytoplasm, large nuclei with prominent nucleoli, small vacuoles, mitochondria, nucleus with prominent nucleoli, plasmodesmata, rough endoplasmic reticulum, chloroplast and the presence of starch grains in embryogenic cells. Zhao et al. (2008) observed that globular somatic embryos composed of cells with dense cytoplasm developed from the surface of the callus prior to differentiate to PLBs. Konieczna et al. (2008) demonstrated that TEM analysis is able to locate the round or oval shaped mitochondria, microbodies, plastids, dicyosomes and numerous ribosomes in the callus of kiwifruit.

Cultured plants with non-functional stomata, weak root systems and poorly developed cuticles caused mortality upon the transfer to *ex vitro* conditions (Mathur et al., 2008). Presence of stomata contributes to the capacity of PLBs to control its water relations. Trichomes are bush-like appendages on the surface of plant tissues, which range in size from a few microns to several centimeters (Tissier, 2012). Theoretically, trichomes may serve as absorption or conduction tube since they are known to accommodate fluids inside.

The dividing cells in developing tissues and organs were smaller and dispensed all cell organs in cytoplasm. The mechanical strength of the cell wall is the main source of structural strength and rigidity for the organs. The majority of plant tissues rely on turgor pressure of the vacuole sap maintaining the cell wall in tension to achieve rigidity. Cuticle is functionally significant for the exchange of water, solutes, gases and the deposition of different substances (Kerstiens, 2006). Cuticles protect the plants against UV radiation, mechanical damage, pathogens and insect. It also provides mechanical strength and contributes to the viscoelastic properties of the cell wall

(Reina-Pinto and Yephremov, 2009). A large number of mitochondria indicate a high level of energy utilization by these cells and is characteristic of tissues undergoing differentiation (Rocha et al., 2012). The frequency of plasmodesmata has been reported increases meristimatic cells because these connections are essential for the intercellular transport of signaling molecules involved in controlling the differentiation pathway of these cells (Appezato-da-Gloria and Machado, 2004). The increased number of Golgi apparatus typically involved in the secretion of substances into apoplast, was associated with an accelerated synthesis of cell wall components in *Glycine max* meristemoids (Steinmacher et al., 2012). Konieczna et al. (2008) pointed out that in embryogenic callus, organelles such as mitochondria and rough endoplasmic reticulum occupied a peripheral position since it affected by large vacuoles.

2.5 Fungal diseases of orchids

Fusarium is an extremely disparaging pathogen that economically limits the production of a number of crops and orchid plants. Frequency of *Fusarium* diseases has been gradually increased in many production amenities worldwide. *Fusarium* has been found to be associated with orchids both as pathogens and non-pathogens. The *Fusarium* species that have been recognized as pathogens includes *Fusarium oxysporum*, *Fusarium proliferatum*, *Fusarium solani*, *Fusarium subglutinans*, and *Fusarium fractiflexum* (Srivastava, 2014).

Fusarium species (*F. proliferatum*, *F. solani*, *F. oxysporum*) cause foliar blight, pseudo-bulb rot and sheath rot on *Dendrobium* orchid (Swett and Uchida, 2015). Wedge and Elmer (2008) reported that *Dendrobium* plantlets affected by fungal disease may die or develop flower and leaves discoloration, and stunted growth leading to more than 50 % yield loss due to *Fusarium* wilt alone. Root discolouration and yellowish stem rotting on *Dendrobium* orchids were strongly associated to *Fusarium* species like *F. oxysporum*, *F. proliferatum* and *F. solani* (Latiffah et al., 2008).

F. oxysporum is a complex and high level of diversified species which has the capability to adapt to any environmental changes and form new pathogenic strains over a short period of time (White et al., 2001; Leslie and Summerell, 2006). *F. oxysporum* is the most extensive and destructive pathogenic fungi of orchid plants. This species is found worldwide such as Australia (Burnett, 1985), India (Yadav et al., 2010; Vijayan et al., 2012), Indonesia (Pinaria et al., 2010), Japan (Ichikawa and Aoki, 2000), Korea (Kim et al., 2002; Lee et al., 2002), Malaysia (Latiffah et al., 2009), Taiwan (Chung et al., 2011; Huang et al., 2014) and United States of America (FUSARlUM Database1.0).

F. oxysporum infects vascular system via roots and resulting deficiencies in nutrients and water intake leading to root rot and wilt diseases (Broadhurst and Hartill, 1996; Ichikawa et al., 2003; Latiffah et al., 2009). Orchid genera susceptible to *F. oxysporum* include *Cattleya*, *Cymbidium*, *Dendrobium*, *Oncidium*, *Phalaenopsis* and *Vanilla* (Kim et al., 2002; Jeong et al., 2004; Pinaria et al., 2010; Pedroso et al., 2011).

2.6 Genetic engineering for orchid development

Since orchids are largely grown for their huge, long-lasting, and attractive flowers, the improvement of certain traits such as flower colour, shelf life, shape, structural design, biotic and abiotic stress tolerance as well as the establishment of novel properties are of essential economic goal for floriculture biotechnologists (Thiruvengadam et al., 2011). Improving orchid plant characteristics via genetic engineering assists the orchid growers to meet the demand of the orchid industry (Teixeira da Silva et al., 2011). *Agrobacterium*-mediated transformation and biolistic are the most popular methods for the development of transgenic orchid plants.

The first successful orchid transformation was reported on *Vanda* (Chia et al., 1990) and *Dendrobium* (Kuehnle and Sugii, 1992; Nan and Kuehnle, 1995) through particle bombardment method. Particle bombardment transformation system was developed in different orchid species like *Cymbidium* (Yang et al., 1999; Chin et al., 2007; Teixeira da Silva and Tanaka, 2009b, 2011), *Dendrobium* (Yu et al., 2001, Chia et al., 2001; Tee et al., 2003; Men et al., 2003a, Tee and Maziah, 2005; Chai et al., 2007; Suwanaketchanatit et al., 2007; Tee et al., 2011), *Oncidium* (Li et al., 2005; Yee et al., 2008), and *Phalaenopsis* (Mishiba et al., 2005).

The very first transgenic *Dendrobium* orchids developed by *Agrobacterium*-mediated transformation system in 1998 (Nan et al., 1998). *Agrobacterium*-mediated transformations were reported for *Cymbidium* (Chin et al., 2007), *Dendrobium* (Yu et al., 2001; Men et al., 2003b), *Oncidium* (Liau et al., 2003a; You et al., 2003; Raffeiner et al., 2009; Thiruvengadam et al., 2012), *Phalaenopsis* (Belarmino and Mii, 2000; Chai et al., 2002; Chan et al., 2005; Mishiba et al., 2005; Sjahril and Mii, 2006; Semiarti et al., 2007; Sreeramanan et al., 2009; Qin et al., 2011), and *Vanda* (Shrestha et al., 2007; Gnasekaran et al., 2014b).

2.6.1 Disease resistant orchid through genetic engineering

Genetic engineering offers a harmonizing approach to conventional breeding programs (Qaim, 2010). The first report on transgenic plants was published in the early 1980s (Zambryski et al., 1983). A variety of transgenic plants have been produced since then. For examples, plants resistant or tolerant to diseases (Delteil et al., 2010;

Harfouche et al., 2011), drought (Harfouche et al., 2011), insect pests (Qaim, 2010), herbicides (Schahczenski and Adam, 2006), low temperatures (Guo et al., 2009; Harfouche et al., 2011) and salinity (Chen and Polle, 2010; Harfouche et al., 2011) as well as possessing an improved nutrient quality (Ye et al., 2000).

Introducing disease resistant gene such as antimicrobial peptides and viral coat proteins (CPs) encoding genes into the genome of some orchids enhanced resistance to *Cymbidium* mosaic virus (CymMV) and *Erwinia carotovora* (Liau et al., 2003b; Chan et al., 2005; Sjahril et al., 2006). Chen et al. (2006) reported that the *Odontoglossum* ringspot virus (ORSV) coat protein gene, green fluorescence protein gene, and hygromycin resistance gene were cloned from ORSV-infected *Epidendrum* to *Cymbidium niveo-marginatum* through *Agrobacterium tumefaciens*. Petchthai et al. (2015) cloned *Cymbidium* mosaic virus coat protein gene into *Dendrobium* orchids and observed transgenic orchids showed viral resistant capacity. Hence, gene transfer techniques may offer a powerful tool to develop disease resistance in transgenic orchid species and it may be feasible to expand this approach to different orchid species (Chin et al., 2007a).

2.7 Selection marker based antibiotic selection technique

Antibiotics inhibit the physiological and biochemical processes in bacteria. Antibiotics attacked mostly five major portions on bacterial cells, such as, cell wall, cell membrane, the synthesis of protein, RNA and DNA synthesis, and metabolism of folic acid (Wright, 2010). Some antibiotics are also able to inhibit protein synthesis in eukaryotes. Kanamycin, geneticin (G-418), neomycin and hygromycin (aminoglycoside antibiotics whose structure is similar to the G-418) are highly effective antibiotics for inhibiting protein synthesis in eukaryotic cells because of their binding capacity to the 80S ribosomal complexes (Mingeot-Leclercq et al., 1999).

The use of suitable selection marker plays an important role in the improvement of transformation efficiency (Opabode, 2006). Selectable marker gene is used to select the transformants and to kill non-transformants explants. Selection marker genes either codes for a protein which detoxify a selection agent or codes for a toxic protein which allows the isolation of transformed plantlets to prevent chimerism and to kill of non-transformed plants (Yu and Xu, 2007; Hsiao et al., 2011). Almost, 50 selection markers have been used as a selective agents for higher plants (Miki and McHugh, 2004), including the genes encoding resistance to antibiotics and herbicides such as neomycin phosphotransferase (*nptII*), hygromycin phosphotransferase (*hpt*), and bialaphos resistance (*bar*). Genes encoding resistance to kanamycin (*nptII*) was effectively used as a selection marker gene for *Cattleya* (Zhang et al., 2010), *Cymbidium* (Chin et al., 2007a), *Dendrobium* (Cao et al., 2006), *Oncidium* (Raffeiner et al., 2009), *Phalaenopsis* (Qin et al., 2011a) and *Vanda* orchids (Gnasekaran et al., 2014b).

2.8 *Agrobacterium*-mediated transformation

The most popular techniques for transgenic orchid production through genetic engineering are *Agrobacterium*–mediated, microparticle bombardment (biolistics) and electroporation (as reviewed by Hossain et al., 2013). Nan et al. (1998) introduced first transgenic *Dendrobium* orchid via *Agrobacterium*-mediated system. Eventually, successful *Agrobacterium*-mediated transformation was reported in *Cattleya* (Zhang et al., 2010) *Cymbidium* (Chin et al., 2007), *Dendrobium* (Yu et al., 2001; Men et al., 2003b; Nie at al., 2005; Cao et al., 2006), *Oncidium* (Liau et al., 2003a; You et al., 2003; Raffeiner et al., 2009; Thiruvengadam et al., 2012), *Phalaenopsis* (Belarmino and Mii, 2000; Chai et al., 2002; Chan et al., 2005; Mishiba et al., 2005; Sjahril and

Mii, 2006; Semiarti et al., 2007; Sreeramanan et al., 2009a; Julkifle et al., 2010; Sreeramanan and Xavier, 2010; Qin et al., 2011a) and *Vanda* (Shrestha et al., 2007; Gnasekaran et al., 2014b).

2.8.1 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a Gram negative soil-borne bacterium which causes crown gall disease and has ability to introduce new genetic material into the plant genome (Gelvin, 2003). Introduced genetic material to the plant genome is called T-DNA (transferred DNA) which is located on a tumor inducing plasmid (Ti- plasmid) (Nester et al., 1984). The Ti-plasmid is disarmed by the deletion of T-DNA and replace with constructed desired gene.

Different *Agrobacterium* strains harbouring various plasmid DNA have been employed in orchids transformation such as LBA4301 (Nan et al., 1998), AGL1 (pCAMBIA1301) (Men et al., 2003b), LBA4404 (pBI121-DOH1as) (Yu et al., 2001) and EHA105 (pSTARGATE-DseDFR470, pWATERGATEDseDFR470, and pWATERGATE-DseCHS-B436) (Ratanasut et al., 2015) in *Dendrobium* genus, EHA 105 (pMT1) (Hsieh et al., 1997), LBA4404 (pTOK233) (Chai et al., 2002), EHA 101 (pEKHWT) (Sjahril et al., 2006) and EHA 101,105 (pCAMBIA1304) (Julkifle et al., 2010; Sreeramanan and Xavier, 2010) in *Phalaenopsis* and LBA4404 (pCAMBIA1304) in *Vanda* orchids (Gnasekaran et al., 2014b), respectively.

2.8.2 *Agrobacterium*-plant interactions

Agrobacterium tumefaciens attach to the surfaces of inanimate objects, plants, and fungi (as reviewed by Li et al., 2012; Matthysse, 2014). Visually, the most important type of attachment of *A. tumefaciens* to surfaces under a diversity of situation is polar attachment. Polar attachment of *A. tumefaciens* is mediated via the unipolar polysaccharide (UPP) (Tomlinson and Fuqua, 2009). However, Aguilar et al. (2011) observed lateral orientation of *Agrobacterium* attachment on a tobacco protoplast. *A. tumefaciens* was observed to significantly respond to callus of sorghum (Verma et al., 2008) and PLBs of *Phalaenopsis* orchid (Sreeramanan et al., 2009a).

Agrobacterium- plant interaction is an excellent concept for studying plant and bacterial responses, as well as the responsibility of chemical signalling in these processes (Brencic and Winans, 2005; McCullen and Binns, 2006; Yuan and Williams, 2012; Pitzschke, 2013). *Agrobacterium* perceives plant-derived signals to stimulate its virulence genes, which are liable for transferring and integrating its T-DNA from its Ti-plasmid into the plant nucleus (Subramoni et al., 2014).

2.8.2.1 *Agrobacterium*-chemotaxis to PLBs

Motility and chemotaxis play a significant role in *Agrobacterium tumefaciens* attachment, biofilm development and virulence (as reviewed by Heindl et al., 2014). In the rhizosphere, *A. tumefaciens* senses plant exudates which in turn induce the virulence gene expression and move towards plant wounds (Shaw, 1991; Chesnokova et al., 1997). A standard laboratory assay for bacterial motility depends on chemotaxis mutants which is created by removal of either the entire chemotaxis operon or the chemotaxis sensor *che A* and impaired for swimming as measured on motility agar plates (Wright et al., 1998; Merritt et al., 2007; Xu et al., 2012). A simple swarm agar plate protocol to study the bacterial chemotaxis is an efficient method to assess the bacteria-plant interaction and the bacterial motility (Perez-Hernandes, 2000; Sreeramanan et al., 2009a).

Agrobacterium has been proven to response chemotactically to wounded PLBs that release acetosyringone; which in turn mediates vir genes induction to initiate T-DNA transfer (Finer, 2010; Gnasekaran et al., 2014b). *Agrobacterium* then initiates infection at the wounded site of host cells (Citovsky et al., 2007). Lengeler (2004) observed that *Agrobacterium* migrated outward from inoculation point by metabolising the nutrient from chemotactic media. Similarly, Monica et al. (2011) found that the *Agrobacterium* chemotactically migrated from inoculation point towards the wounded plant cells. Julkifle et al. (2012) also studied on bacterial chemotaxis response of *Dendrobium* sonia-28 orchid PLBs and reported that *Agrobacterium* showed positive chemotaxis response towards wounded *Dendrobium* sonia-28 orchid's PLBs.

2.8.2.2 *Agrobacterium* infection process

Agrobacterium pathogenicity is recognized for the evolved prospective of precise detection and response to plant derived chemical signals (as reviewed by Subramoni et al., 2014) such as neutral and acidic sugars, and phenolic compounds. Successful colonization of *Agrobacterium* lead to the transfer of T-DNA (Transferred-DNA) from the T-region on Ti- plasmid of *Agrobacterium* to the plant cell which codes for the synthesis of opines (crown gall-specific molecules synthesized by transformed plants), and *vir* (virulence) proteins (Zhu et al., 2000). Ti plasmids (tumor inducing-plasmid) are on the order of 200 to 800 kbp in size while T-DNA averages between 10 to 30 kbp (Suzuki et al., 2000).

The T-DNA locates oncogenic genes coding for the virulence (*Vir*) proteins that assist in the T-DNA transfer, nuclear targeting, and integration into the plant genome (Gelvin, 2003; Gelvin, 2012; Pitzschke, 2013). Successful T-DNA incorporation into plant nuclei made possible by establishing different host system, cytoskeletal networking, defence signalling, molecular motors, nuclear import, proteolytic degredation, chromatin targeting, and repair to ensure successful plant transformation (as reviewed by Citovsky et al., 2007).

2.8.2.3. Reporter genes

A good reporter gene selection is an important component for the success of genetic engineering in plants (Basu et al., 2004). Firstly, reporter gene is used as a substitute of a gene that confers a desirable characteristic for the optimization purposes in transformation studies (Rosellini, 2012). Early detection of plant transformation trials is required for the optimization of transient and stable gene transfer in a plant genome (Teixeira et al., 2011). Different types of reporter gene such as *gusA* which codes for β-glucuronidase (GUS) (Oyelakin et al., 2015), green fluorescent protein (GFP) which was found in jellyfish (Takata and Taniguchi, 2015), red fluorescent protein (DsRED) from *Discosoma sp.* (Zhang et al., 2015), firefly luciferase gene (*luc*) (Chia et al., 1994) and Chloramphenicol acetyltransferase (CAT) which was bacterial enzyme (Bronstein et al.,1994) were usually used for the optimization purposes in plant transformation studies.

The GUS is the most commonly used reporter gene or marker used in transformation studies, which gives the blue colour of transformed cells through the synthesis of β-glucuronidase by catalyzing the exogenously uses of X-gluc (Jefferson et al., 1987). The transient GUS assay has been used as a preliminary confirmatory study for transformation of numerous plant species such as Arabidopsis (Wu et al., 2014; Han et al., 2015), banana (Rustagi et al., 2015), brinjal (Kumari et al., 2013), cassava (Oyelakin et al., 2015), cotton (Yu et al., 2015), groundnut (Tiwari et al.,