Emir. J. Food Agric. 2010. 22 (2): 103-116
http://ffa.uaeu.ac.ae/ejfa.shtml

Early GFP gene assessments influencing *Agrobacterium tumefaciens*-mediated transformation system in *Phalaenopsis violacea* orchid

Sreeramanan Subramaniam[1*] and Zuraida Abdul Rahman[2,3]

[1]School of Biological Sciences, Universiti Sains Malaysia (USM), Minden Heights, 11800, Penang, Malaysia, [2]Biotechnology Research Centre, MARDI, G.P.O Box 12301, 50774, Kuala Lumpur, Malaysia; [3]Biotechnology Research Centre, MARDI Headquarter, Persiaran Mardi-UPM, 43400 Serdang, Selangor, Malaysia

Abstract: An initial step in the genetic transformation of *Phalaenopsis violacea* orchid species was investigated in the plant-*Agrobacterium* interaction. *Agrobacterium tumefaciens* strains EHA 101 and 105, harboring the pCAMBIA 1304 plasmid contains *gfp* gene as the reporter gene marker, were used in this transformation study. The spectrophotometric GFP assay provides information on the amount of inoculated *Agrobacterium tumefaciens* that effectively bound to various orchid tissues. Different temperatures during co-cultivation period, the concentration of L-cysteine, calcium ($CaCl_2$) and silver nitrate ($AgNO_3$) in co-cultivation medium during co-cultivation period were identified to be major and important factors in enhancing the increase percentage of transient *gfp* gene expression in PLBs. *Agrobacterium tumefaciens* EHA 105 was proved to be better bacterial strain in transforming the targeted PLBs than EHA 101, based on the notably higher transient expression of *gfp* gene in all the optimization parameters that were tested. Highest T-DNA delivery efficiencies were obtained when *P. violacea* PLBs were co-cultivated with *Agrobacterium tumefaciens* strain EHA 105 in half-strength MS medium supplemented with 5% of banana cultivar, Mas extract containing 200 mgL^{-1} L-cysteine, 60µM silver nitrate, without calcium in the medium during co-cultivation in the dark condition at 24°C. The results from the transient *gfp* gene expression of PLBs suggested that *Agrobacterium*-mediated transfer of T-DNA to the naturally recalcitrant *P. violacea* is feasible and is highly efficient. Hence, the use of the *gfp* marker gene during *in vitro* screening of the transgenic cells has enabled the visual selection of orchid transformed by *Agrobacterium tumefaciens* at higher frequency rates.

Keywords: *gfp* gene, *Agrobacterium tumefaciens*, *Phalaenopsis violacea*, orchid.

التقييم الأولي بواسطة **GFP** لدراسة تاثيرها ***Agrobacterium tumefaciens*** بوساطة التحول في النظام ***Phalaenopsis violacea*** من فصيله الاوركيد

سرى رمنن صبرا منيم[1]*، و زوريدا عبدالرحمن [2,3]

مدرسة علوم الحياء، جامعة صينص ماليزيا(USM) منتن هيتس، 11800، بننك، ماليزا، 2 مركز بحوث التكنولوجيا الحيوية، MARDI, G.P.O Box 12301, 50774, **كوالالامبور، ماليزيا،3 مركز بحوث التكنولوجيا الحيوية،** MARDI **المقر الرئيسي، برسيارن ماردي** – UPM, **43400سيرتانق، سلانقور، ماليزيا**

ملخص: تعتبر هذه خطوة اولى في دراسه التحول الجيني لنبات الفالانوبسيس فيولاسيا من فصيله الاوركيد وتفاعله مع نبات الاجروباكتيروم . نوعان للاجروباكثيريوم تيومفاشينز هما EHA 101و105 ويحتويان على pCAMBIA 1304 plasmid ويحمل جينة gfp التي استخدمت في هذه الدراسة. قدمت GSP معلومات عن كمية وجود الاجروباكثيريوم تيومفاشينز المتصلة لعدد متنوع من انسجة الاوركيد. اختلاف درجات الحرارة خلال فترة الرعاية، كميات تركيز مادة L-cysteine و كالسيوم (CaCl2)، ونترات الفضة(AgNO3) اعتبرت من أهم العوامل لزيادة نسبة جين gfp المدخل من خلال اسلوب PLBs. الاجروباكثيريوم تيومفاشينز نوع EHA 105 اثبت انه افضل انواع البكتيريا عند تحويل PLBs المطلوبه عن نظيره EHA 101، وذلك حسب وجود جين gfp المتحول في كل وحدات القياس الخاضعه للاختبار.أعلى أداء لـ T-DNA بفعالية رصدت عندما كان نبات البي فيولاسيا PLBs يعيش على نوع الاجروباكثيريوم تيومفاشينز EHA 105 وذلك في نصف قوة الوسيط ام اس المزودة بـ خمسه بالمئه من banana cultivar ، و 200 $mg.L^{-1}$ L-cysteine، و 60µM silver nitrate ، وبدون كالسيوم وفي وسط مظلم بدرجة 24°C. النتائج من جين gfp المدخل من خلال اسلوب PLBs توحي بان الاجروباكثيريوم

* Corresponding Author, *Email*: sreeramanan@gmail.com

المتحول عن T-DNA إلى بي فيولاسيا الطبيعية يعتبر معقول وفعال بدرجة كبيرة . ولذلك استخدام جين gfp خلال فصل الخلايا المتحولة سمحت بالاختيار النظري لنبات الاوركيد المتحول بسبب الاجروباكثيريوم تيومفاشين بمعدلات اعلى .

Introduction

Phalaenopsis violacea have greenish white flowers with purple pigment around the sepals and the lip. *P. violacea* are native to the peninsular Malaysia and are closely related to *P. bellina* of the Borneo. *P. violacea* are important parental varieties to produce novel *Phalaenopsis* hybrids with special fragrance. In order to improve the quality, modern molecular biology techniques could be applied to transfer desired gene(s) into orchid genome instead of using conventional breeding method which is time consuming and lack of genetic variability.

Currently, *Agrobacterium tumefaciens* mediated gene transfer system, microprojectile bombardment and direct gene transfer into the orchid protoplast, are the three major gene transformation methods applied in orchid biotechnology. However, *Agrobacterium* mediated transformation has several advantages over other direct methods such as the transfer of relatively large segments of DNA with little rearrangement and the integration of low copy numbers of T-DNA into transcriptionally active regions of the chromosome and unlinked integration of co-transformed T-DNA (Fang et al., 2002; Olhoft et al., 2004; Lee et al., 2006; Ying et al., 2006). Attachment of *Agrobacterium tumefaciens* to target plant cells are essential for tumorigenesis and appear to be mediated by specific receptors located on the bacterial and plant cell surface. *Agrobacterium tumefaciens* binds to the plant cell in a two step process, in which an initial loose attachment of individual bacterial cells is followed by a tight binding and massive aggregation of bacteria at the host cell surface (Perez Hernandez et al., 1999).

Attachment of *Agrobacterium tumefaciens* to plant cells can be observed through a number of microscopy techniques with the specificity of the cell–cell contact would be preferably demonstrated by a quantitative measurement of the binding capacities of attachment competent bacteria (Kado, 1998; Vergauwe et al., 1998). It is believed that the tight attachment of agrobacterial cells to the surface of plant cells is due to microfibrils containing cellulose, a linear plant polysaccharide composed of glucose residues linked by β-1,4 bonds (Gurlitz et al., 1987). The present experiment describes the study of *Agrobacterium* attachment to *Phalaenopsis violacea* cells and tissues, using the quantification of bacterial attachment through the spectrophotometric GFP expression assay.

Early detection of plant transformation events is necessary for the optimisation of transient and stable gene transfer into orchid genome. Green fluorescent protein (GFP) is a convenient marker as it is absent in most wild type organisms unlike some plant species which posses their own *gusA* gene (GUS expression), thus, interfering in the selection of transformants. Apart from that, GFP is a practical genemarker as it is easy to be observed, non-destructive, and cell autonomous which does not require extragenous substances for fluorescence to occur with the integrity of the target tissue are maintained (Elliot et al., 1999; Jordan, 2000). Transient *gfp* gene expression was used to evaluate the efficiency of T-DNA delivery in transformants due to its simple, non-destructive and cell autonomous procedure (Jordan, 2000; Fang et al., 2002).

Protocorm-like bodies (PLBs) were used as a starting material in the transformation work since it is an activated tissue with the presence of coniferyl alcohol which could induce *vir* genes in orchids (Nan et al., 1997) and rapidly multiply, proliferate in a shorter period. In

104

the present study, temperatures during co-cultivation period, the concentration of L-cysteine, calcium ($CaCl_2$) and silver nitrate ($AgNO_3$) in co-cultivation medium conditions, were identified as important factors in enhancing the percentage of transient *gfp* gene expression in *P. violacea* orchid. Hence the identification and studies of such factors of this process hold great promise for the future genetic transformation of various orchid plant species and hybrids, as they might help in the development of conceptually new techniques and approaches needed today to expand the host range of *Agrobacterium tumefaciens* and to control the transformation process precisely.

Materials and Methods

Plant materials

Phalaenopsis violacea wild orchid plants were obtained from Mr. Michael Ooi's orchid nursery in Seberang Jaya, Penang (Figure 1). The *P. violacea* PLBs were obtained from young segments of approximately 1 x 1 cm^2, excised from aseptically raised three-month old *in vitro* seedlings. For quantification of *Agrobacterium* attachment experiment, roots, PLBs, and shoot tips were used. PLBs of *Phalaenopsis violacea* were obtained from *in vitro* plantlets of three months culture using ½ strength of Murashige and Skoog (1962) medium supplemented with 5% of banana cultivar, Mas (AA) extract. Cultures were incubated on tissue culture room at 25^oC under 16 hours photoperiod with light intensity of 40 $\mu mol.m^{-2}.s^{-1}$ supplied by white fluorescent tubes. Proliferated PLBs after 2 months were used for the *Agrobacterium*-mediated transformation experiment.

Figure 1. The appearance of *Phalaenopsis violacea* orchid plant. The bar in the bottom image represents 1.0 cm.

***Agrobacterium tumefaciens* strains and plasmid DNA**

Agrobacterium tumefaciens super-virulent strains, EHA 101 and 105 harbored disarmed pCAMBIA 1304 plasmid with green fluorescence protein (*gfp*) gene. The plasmid is driven by *CaMV 35S* promoter and contained *hpt* gene for resistance to the hygromycin antibiotic. Three mL cultures form the stock were transferred into Luria-Bertani (LB) medium containing 50 mgL^{-1} kanamycin and incubated at 28ºC and 120 rpm overnight to reach an optimal density. 500µL of the bacterial suspension were streaked on solid LB medium containing 50 mgL^{-1} kanamycin and incubated at 28ºC for 2-3 days. Single colony were then grown in LB medium containing 50 mgL^{-1} kanamycin and incubated at 28ºC and 120rpm for 16 hours to reach an optimal density between 0.5-0.75 units at 600nm (OD_{600nm}).

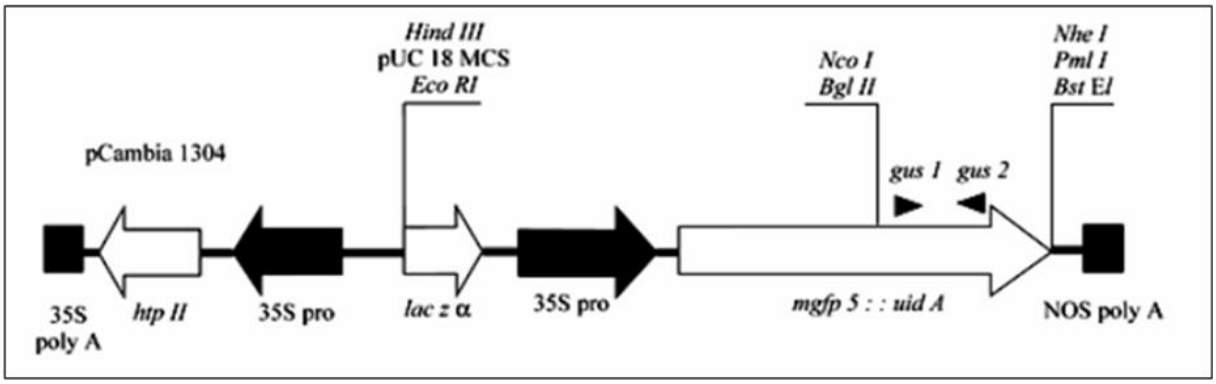

Figure 2. Schematic diagram of the plasmid pCAMBIA 1304. The binary vector pCAMBIA 1304 (CSIRO, Australia) harboring the reporter *gusA* and *mgfp5* genes driven by the *CaMV* 35S promoter.

Quantification of bacterial attachment to *Phalaenopsis violacea*'s root, protocorm -like bodies (PLBs) and shoot tip through spectrophotometric measurement of GFP expression in genetically marked *Agrobacterium tumefaciens* strains

Quantification of bacterial attachment assays were carried out according to method of (Perez Hernandez et al., 1999). Root, PLBs (3-4mm) and shoot tip were prepared. During preparation, explants were maintained in 25 mM phosphate buffer (pH 7.5). For infection, 1.5 ml Eppendorf tubes filled with 1 ml of the same buffer were loaded with 50µL aliquots of buffer suspended bacteria. Tubes were then incubated in a rotary shaker at 28ºC at 25 rpm for 2 hours. After this period, unbound bacteria were removed by washing the explants 2-3 times with 1 ml fresh buffer and vortexed 60 seconds each time to discard unattached bacteria. Green fluorescent protein (GFP) activity in the samples was measured following the β-glucuronidase activity as mentioned above except there is no substrate was used and the activity quantified by measuring direct light absorbance at 510 nm (A_{510nm}) as described by Remans et al. (1999). Finally, the percentage of inoculated bacteria that remained attached to the different tissues (% Att) was calculated using the formula:

$$\% \text{ Att} = (X - Y) \times 100 / Z$$

where the variables are the absorbance values corresponding to infected tissues (X), uninfected tissues (Y) and total bacterial inoculum (Z) for each individual combination of explants type and bacterial strain.

Experimental design: Optimization of various parameters affecting *gfp* gene transfer mechanisms

To assess factors affecting the transformation efficiency in *P. violacea* PLBs, four different parameters were performed. The parameters were included different temperatures for gene transfer (20, 22, 24, 26, 28, 30, and 32ºC), various L-cysteine concentrations (0, 100, 200, 300, 400, 500, and 600 mgL^{-1}), different calcium strengths (0, 0.25, 0.5, 0.75, 1.0 and 1.5 strengths), and different silver nitrate concentrations (0, 20, 40, 60, 80, 100, and 120 µM) during co-cultivation period. A range of parameters were evaluated with each experiment containing 30single PLBs per replicate. To determine the optimum conditions for transformation, one factor of the standard conditions was changed each time and the effects on percentage of transient *gfp* gene expression were evaluated.

Green fluorescent protein (GFP) histochemical assessment

Transient expression levels of *gfp* gene in the PLBs were assessed three days after co-cultivation period using a stereomicroscope equipped for epi-fluorescence illumination (Leica MZFLIII). Transformation frequency was calculated as the percentage of PLBs expressing GFP over the total number of inoculated PLBs.

Statistical analysis

Data were analyzed using one-way ANOVA in SPSS 10.0 (SPSS Inc. USA). All analyses were performed at a significance level of 5% with the differences contrasted using Duncan's multiple range test.

Results and Discussion

Quantification of bacterial attachment

Besides the chemotaxis assay (Shaw, 1995; Sreeramanan et al., 2009), a system for the quantification of bacterial attachment was developed, which provided information about (i) the specificity of the process for attachment–component *Agrobacterium* (ii) the amount of inoculated bacteria that effectively bound to plant cells. Microscopic examination of bacteria interacting with the plant cells indicates a significant propensity to attach in a polar fashion (Smith and Hindley, 1978). Quantitative estimation of binding by *Agrobacterium* to plant cells has revealed two types of interactions: a nonspecific, non-saturable, aggregation-like interaction readily removed via washes with a buffered salt solution and a specific, saturable interaction (200–1000 bacteria per plant cell) impervious to such washing (Gurlitz et al., 1987). Therefore, it would be of interest to know whether the same pattern can be found during *Agrobacterium*-orchid interaction, before attempting transformation in this species. The spectrophotometric GFP assay used for quantification of bacterial attachment revealed the increased binding ability of the attachment – efficient *Agrobacterium* strains, EHA 101 and 105 (Figure 3).

Significant differences were observed among the two *Agrobacterium* strains tested with the attachment to protocorm-like bodies of *P. violacea* compared with the other explants (Figure 3). This could be due to protocorm like-bodies segments contain the highest exposed cell surface of all explants tested and thus provide the most numerous binding sites for an effective attachment of competent bacteria. In the cases of other types of explant, the proportion of intercellular spaces where bacteria could refugee and escape from washings is increased with respect to the available sites for an effective binding, diminishing the differences between binding–deficient and efficient bacteria for colonizing these tissues.

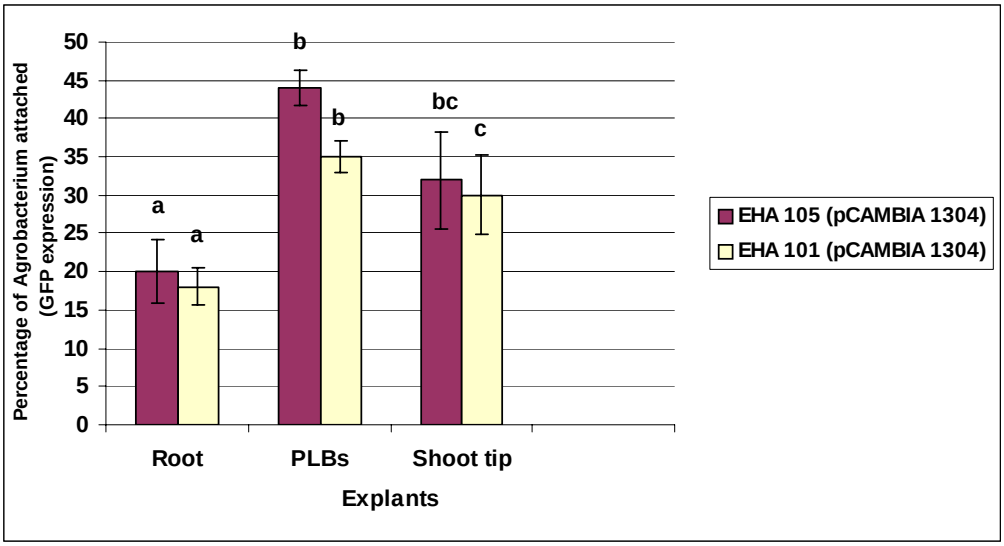

Figure 3. Quantification of bacterial attachment to *Phalaenopsis violacea* PLBs, shoot tip, leaf and root explants through spectrophotometric measurement of GFP expressions in genetically marked bacteria. Values correspond to the percentages of inoculated bacteria remain attached to cells after extensive washing of infected tissues. Data were analyzsed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$).

Therefore, PLBs provide a better system for studying bacterial attachment to plant cells. Using this type of tissue, bacterial attachment to wheat, maize, pea and banana cells could be also quantified, illustrating the applicability of the system to the study of *Agrobacterium* attachment to other plant species (Perez Hernandez et al., 1999; Sreeramanan et al., 2006). Similarly to what was observed in the case of *P. violacea* orchid, the superior binding ability of the attachment–competent *Agrobacterium* strains was also evidenced (Figure 3).

Optimisation of gene transfer using two different hypervirulent *Agrobacterium tumefaciens* strains

Agrobacterium strains EHA 101 is derived from the supervirulent wild-type strain A281 and subsequent removal of kanamycin resistance gene from the bacterial chromosomes derived strain EHA 105. These supervirulent derivatives are highly efficient in gene transfer. The agropine-type hypervirulent strain EHA 105 was proved to be more efficient in gene transfer compared to the octopine-type (i.e. strain LBA 4404) and nopaline-type (i.e. strain GV 3101) in *Zea mays* L. (Huang and Wei, 2005). In comparison between strain LBA 4404 and EHA 101, it was shown that successful wheat transformation was facilitated by EHA 101 (McCormac et al., 1998; Karami, 2008). In our experiments, T-DNA delivery was higher in EHA 105 assisted transformation than EHA 101 in all evaluated factors. This is probably due to the increase induction of *vir* genes, by *vir*G and *vir*A genes of the disarmed pTiBo542 of the EHA 105 (Gelvin, 2003; Karami, 2008). Previously, we have reported EHA 105 demonstrate faster migration of positive chemotaxis response and has higher bacterial attachment to *P. violacea* compared to EHA 101 (Sreeramanan et al., 2009).

Naturally, monocotyledonous orchid plants are not a host for *Agrobacterium tumefaciens* infection. The choice of *Agrobacterium* strains plant an important important role in the transformation process for the efficiency of gene transfer on recalcitrant species of orchid such as *P. violacea* in this study. Effectiveness of T-

DNA delivery into PLBs was evaluated based on the percentage of transient *gfp* gene expression after three days. Two *Agrobacterium* strains (EHA 101 and EHA 105) were compared for their level of competency to *P. violacea*. Generally, there is no significant different in the transformation efficiency between the both *Agrobacterium tumefaciens* strains. However, EHA 105 display higher *gfp* gene expression for all the parameters tested compared to EHA 101.

Different temperature conditions

Temperature has been considered a factor affecting the capacity of *Agrobacterium* to transfer the T-DNA to plant cells (Karami, 2008). The optimal temperature for both transient and stable transformation events should be evaluated with each specific explants and *Agrobacterium tumefaciens* strains involved (Sales et al., 2001). The effect of different temperatures for gene transfer on transient *gfp* gene expression in *Phalaenopsis violacea* PLBs were tested at the range of 20-32ºC with 2ºC intervals. The highest gene transfer was observed at 24˚C with no significant difference ($p<0.05$) between both strains (Figure 4). Transformation efficiency was greatly reduced at lower and higher temperature.

Based on the transient *gfp* gene expression, temperature is a vital parameter during gene transfer. Low temperature of 24°C for co-cultivation of *Agrobacterium* has been optimized for other monocots such as Alstromeia plants (Kim et al., 2007) and dicots such as tomato (Ahsan et al., 2007). Low temperatures were suggested to improve pili number on cell surface for better attachment and better functioning of the *vir*B-*vir*D4 (Fullner et al., 1996), hence, the enhancement of transformation. Higher transformation frequency was observed in maize immature embryo transformation at 20ºC than at 23ºC when using a standard binary vector (Frame et al., 2002). In contrast to the results of Dillen et al. (1997) which reported that temperatures of 25 and 28ºC yielded significantly greater kenaf shoot apex survival on selection medium than 16 and 19ºC.

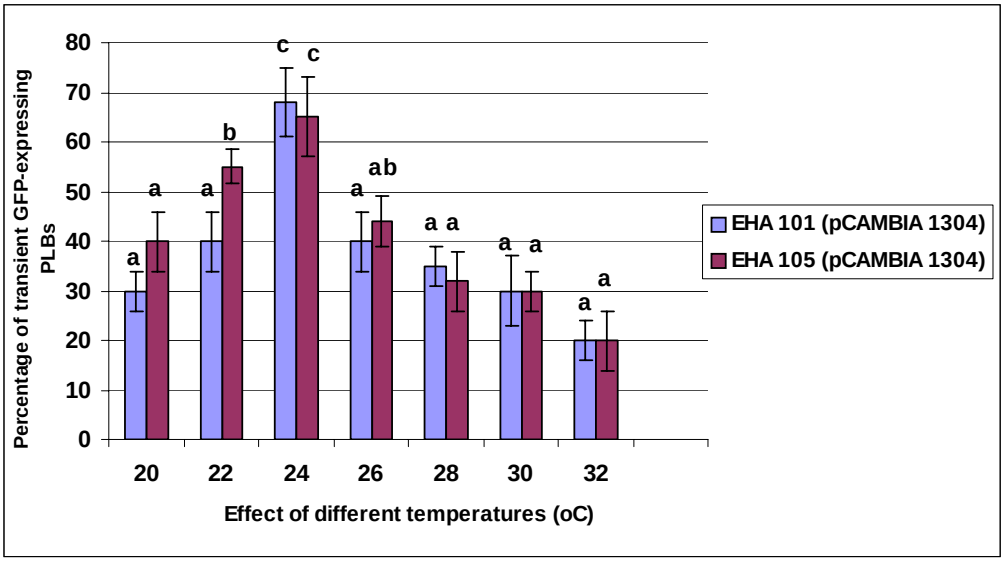

Figure 4. Effect of the different temperatures for gene transfer on transient *gfp* gene expression in *Phalaenopsis violacea* PLBs. For each parameter, three replicates were used containing ten PLBs per replicate and were repeated three times. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$).

Different concentrations of L-cysteine

L-cysteine is an amino acid, with a thiol side chain, important component of antioxidant glutathione. L-cysteine antioxidant effect on *Agrobacterium*-mediated transformation has been studied intensively in soybean varieties (Olhoft et al., 2001; Paz et al., 2004; Liu et al., 2008). While evaluating for effect of different concentrations of L-cysteine supplement in the co-cultivation media, it was found that the highest expression of *gfp* gene is at 200 mgL^{-1} of L-cysteine (Figure 5). Higher concentrations of L-cysteine (more than 200 mgL^{-1}) result in less transient *gfp* gene expression and browning on PLBs, similar to those symptoms caused by hypersensitive response.

L-cysteine supplement in the co-cultivation media was notably assist gene transfer in *P. violacea* in both strains. L-cysteine is an effective inhibitor for polyphenol oxidases (PPOs), peroxidases (PODs) and enzymatic browning. It has been reported that with the addition of L-cysteine could significantly increases transformation efficiency, especially in combination of other thiol compounds (Olhoft et al., 2001) or surfactant (Liu et al., 2008). The authors also suggested that L-cysteine improve transformation by reducing plant defense response to pathogen attack, plant wounding and environmental stresses throughout co-cultivation period. L-cysteine therefore reduced plant cell death, enzymatic browning of wounded sites, and increase bacterial susceptibility which subsequently improved transformation efficiency. However, higher L-cysteine concentration results in browning of the PLBs, similar to those observed in hypersensitivity symptoms. It is possible that at high concentration, the explants utterly loss its capability to recover itself from the wounding stress and pathogen attack hence causing death and reduced percentage of transformation.

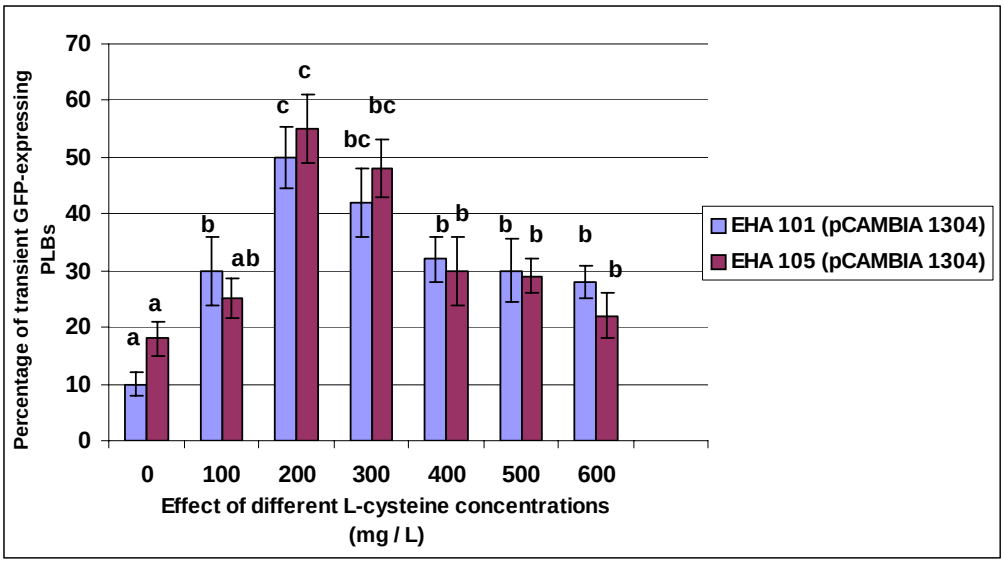

Figure 5. Effect of the L-cysteine concentrations on transient *gfp* gene expression in *Phalaenopsis violacea* PLBs. For each parameter, three replicates were used containing ten PLBs per replicate and were repeated three times. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$).

Different strengths of calcium

Calcium is important for structural roles in cell wall and membranes. Calcium ion directly acts as an ionic cross-linkage of the carboxyl groups of linear macro molecule in the cell wall. Deficiencies of calcium lead to deterioration of cell membrane resulting in cells become leaky,

modified with consequently reduced the cell wall matrixs, opposing *Agrobacterium* cell attachment. Therefore, presence of calcium during co-cultivation period confers adverse effect on transient expression of *gfp* gene in PLBs. T-DNA transfer was significantly ($p<0.05$) the most efficient at the absence of (0 mgL^{-1}) calcium (Figure 6). Strains, EHA 101 and EHA 105, recorded 65% and 70% transformation rate, respectively, in the calcium-free co-cultivation medium. As the concentration increases, gene transfer by both *Agrobacterium* strains decreases dramatically.

Calcium also required in regulating various secondary messengers coordinating numerous developmental signals as well as changes in cell status in respond to environmental and disease challenges. It is observed that calcium deprived co-cultivation media enhanced *Agrobacterium*-mediated transformation. Calcium-free medium was proved to deplete 91% of endogenous calcium in *Hevea brasiliensis* calli (Montoro et al., 2000) which initially reduce the cell wall matrix due to the changes on cell wall structure and finally, increase T-DNA delivery into the plant cell (Montoro et al., 2003).

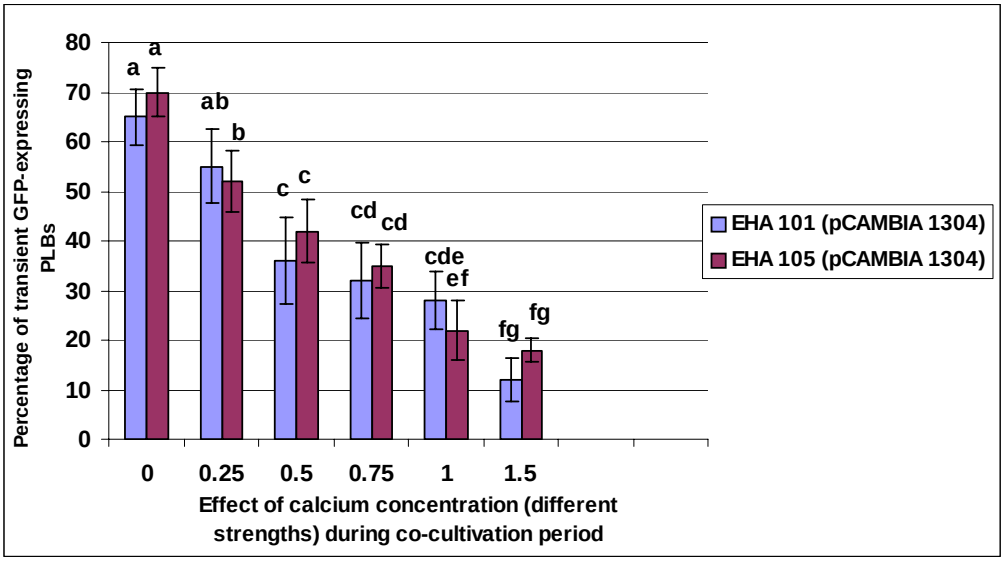

Figure 6. Effect of the different calcium strength during co-cultivation period on transient *gfp* gene expression in *Phalaenopsis violacea* PLBs. For each parameter, three replicates were used containing ten PLBs per replicate and were repeated three times. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$).

Different concentrations of silver nitrate

Silver nitrate compound is known to inhibit ethylene production from the *in vitro* culture, which affects plant cell growth mechanisms. Generally it is known that silver nitrate will suppress the ethylene biosynthesis pathways via Ag^{+} reducing capacity to bind various ethylene receptors. In this study, the effect of different silver nitrate concentrations on *Phalaenopsis violacea* PLBs for determination of transformation efficiency were examined based on the percentage of transient GFP expression. Different silver nitrate concentrations in co-cultivation media were found influencing *gfp* gene transfer into the PLBs explants. It was observed that, 80% and 71% of PLBs

transformed with EHA 105 and EHA 101, respectively, were *gfp* positive in medium containing 60µM silver nitrate (Figure 7). Higher and lower silver nitrate concentration significantly ($p<0.05$) downsized the number of transformants.

Similar to L-cysteine, silver nitrate is also an antioxidant compound. Enriquez-Obregon et al. (1997) reported that competence of sugarcane plant tissue to the *Agrobacterium*-mediated gene transfer was improved with the appropriate addition of antioxidant compounds, including silver nitrate, cysteine and ascorbic acid. They observed that, cell death due to hypersensitivity reaction after cutting was decreased with the addition of these antioxidant compounds. At higher concentration (more than 60µM) of silver nitrate, the gene transfer severely decreased due to accumulation of phenolic compounds in the PLBs. Tao and Li (2006) also observed the same phenomenon of increased transformation efficacy of *Torenia fournieri* in low concentration and decreases at high concentration of silver nitrate. Hence, silver nitrate significantly suppresses the *Agrobacterium* growth during co-culture without any effect on the T-DNA delivery and integration into the orchid genome. In addition, the suppressed of *Agrobacterium* growth on the PLBs could facilitate plant cell recovery and improved the regeneration process (Opabode, 2006).

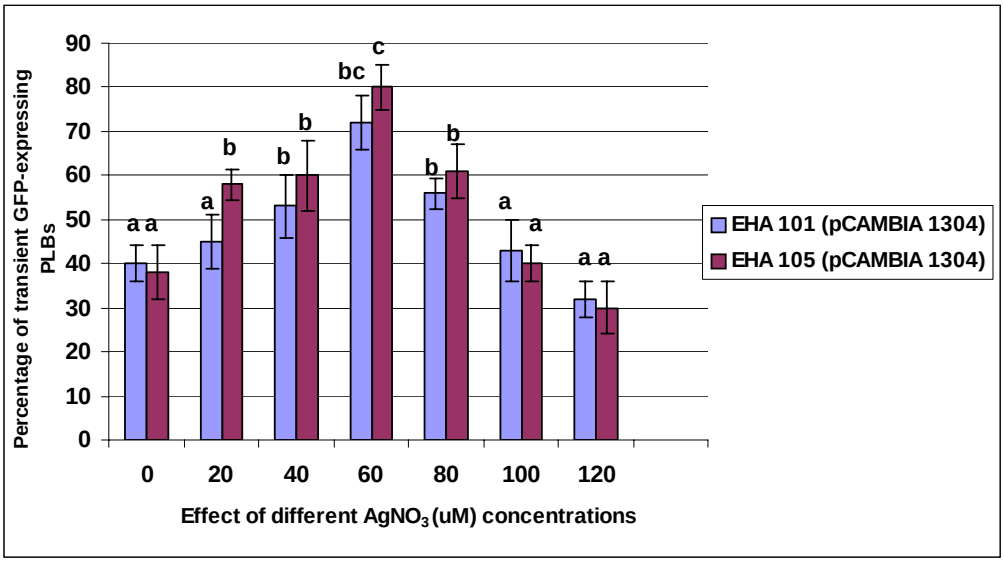

Figure 7. Effect of the silver nitrate concentrations on transient *gfp* gene expression in *Phalaenopsis violacea* PLBs. For each parameter, three replicates were used containing ten PLBs per replicate and were repeated three times. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$).

Conclusion

The quantification system proved that *Agrobacterium* is able to attach specifically to different types of *P. violacea* orchid cells based on the GFP spectrometric assay. GFP can provide a simple yet powerful tool for optimizing *Agrobacterium tumefaciens* colonization and infection of plant tissues resulting in increased transformation frequency of plant tissue. Once *Agrobacterium* reaches the neighbourhood of wounded tissues, the next step required for the development of plant tumors is its attachment to plant cells. *Agrobacterium* was able to bind to wounded as well as to unwounded plant cell surfaces, questioning the long debated requirement of plant cell damage for

transformation, at least during the initial phase of bacterial colonization. In addition, the binding process was quantified, which provided a further evidence for the specific ability of virulent *Agrobacterium* to colonize tissues from PLBs of *P. violacea.* Tissue browning is apparently a part of plant defense machinery which makes a dead cell barrier around the wounded sites to protect plants from further spread of injury. The choice of starting material has proved to be crucial in successful *Agrobacterium*-mediated orchid transformation. The results obtained in the second phase of the study revealed that successful transformation of *Agrobacterium tumefaciens*–mediated system in *P. violacea* PLBs is due to the optimization of major key factors: the concentration of L-cysteine, calcium ($CaCl_2$) and silver nitrate ($AgNO_3$) in co-cultivation media during co-cultivation period. The optimization of these factors is a critical step as it breaches the limitation of *Agrobacterium* T-DNA delivery into recalcitrant species, such as *P. violacea* orchid. It is also demonstrated that transient *gfp* gene expression of transformants can be used with high reliability and efficacy to detect and isolate transformants PLBs. The use of various other *Agrobacterium tumefaciens* strains and the combination with super binary vectors and binary vectors with a constitutively active *virG* may further improve transformation efficiency in many or all orchid's species and hybrids. The established protocol for *Agrobacterium*-mediated transformation here therefore, can be utilised for gene-of-interest transfer into *P. violacea* and its related orchid species.

Acknowledgement

The authors wish to thank Dr. Richard I.S. Bretell from CSIRO, Australia for the pCAMBIA 1304 plasmid. This research was supported by Research University Grant (Genetic Engineering of *Dendrobium* hybrid orchid for fungal disease resistance) from the Universiti Sains Malaysia (USM).

References

Ahsan, N, S. H. Lee, D. G. Lee, M. Anisuzzaman, M. F. Alam, H. S. Yoon, M. S. Choi, J. K. Yang and B.H. Lee. 2007. The effects of wounding type, pre-culture, infection method and co-cultivation temperature on the *Agrobacterium*-mediated gene transfer in tomatoes. Ann. Appl. Biol. 151:363-372.

Dillen, W., J. De Clercq, J. Kaplia, M. Zambre, M. Van Montagu and G. Angenon. 1997. The effect of temperature on A*grobacterium tumefaciens*-mediated gene transfer to plants. Plant J. 12:1459-1463.

Elliot, A. R., B. Campbell, B. Dugdale, R. I. S. Brettell and C. P. L. Grof. 1999. Green fluorescent protein facilitates rapid *in vivo* detection of genetically transformed plant cells. Plant Cell Rep. 18:707-714.

Enriquez-Obregon, G. A., R. I. Vazquez-Padron, D. L. Prieto-Samsonov, M. Perez and G. Selman-Housein. 1997. Genetic transformation of sugarcane by *Agrobacterium tumefaciens* using antioxidant compounds. Biotecnol. Apl. 14:169-174.

Fang, Y. D., C. Akula and F. Altpeter. 2002. *Agrobacterium* mediated barley (*Hordeum vulgare* L.) transformation using green fluorescent protein as a visual marker and sequence analysis of the T-DAN::barley genomic junction. J. Plant Physiol. 159:1131-1138.

Frame, B. R., H. Shou, R. K. Chikwamba, Z. Zhang, C. Xiang, T. M. Fonger, S. E. K. Pegg, B. Li, D. S. Nettleton, D. Pei and K. Wang. 2002. *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol. 129:13-22.

Fullner, K. J., J. C. Lara, E. W. Nester 1996. Pilus assembly by *Agrobacterium* T-DNA transfer genes. Sci. 273:1107-1109.

Gelvin, S. B. 2003. *Agrobacterium*-mediated plant transformation: the biology behind the 'gene jockeying' tool. Microbiol. Mol. Biol. Rev. 67:16-37.

Gurlitz, R. H. G., P. W. Lamb, A. G. Matthysse. 1987. Involvement of carrot surface proteins in attachment of *Agrobacterium tumefaciens*. Plant Physiol. 83:564–68.

Huang, X., Z. Wei. 2005. Successful *Agrobacterium*-mediated genetic transformation of maize elite inbred lines. Plant Cell Tissue Organ Cult. 83:187-200.

Jordan, M. C. 2000. Green fluorescent as a visual marker for wheat transformation. Plant Cell Rep. 19:1069-1075.

Kado, C. I. 1998. *Agrobacterium*-mediated horizontal gene transfer. Gen Eng. 20:1-24.

Karami, O. 2008. Factors affecting *Agrobacterium* mediated transformations of plants. Trans Plant J. 2:127-137.

Kim, J. B., C. J. J. M. Raemakers, E. Jacobsen and R. G. F Visser. 2007. Efficient production of transgenic *Alstromeria* plants by using *Agrobacterium tumefaciens*. Ann. Appl. Biol. 151:401-412.

Lee, S. H., D. G. Lee, H. S. Woo, K. W. Lee, D. H. Kim, S. S. Kwak, J. S. Kim, H. Kim, N. Ahsan, M. S. Choi, J. K. Yang and B. H. Lee. 2006. Production of transgenic orchardgrass via *Agrobacterium* mediated transformation of seed derived callus tissues. Plant Sci. 171:408-414.

Liu, S. J., Z. M. Wei and J. Q. Huang. 2008. The effect of co-cultivation and selection parameters on *Agrobacterium* mediated transformation of Chinese soybean varieties. Plant Cell Rep. 27:489-498.

McCormac, A. C., H. Wu, M. Bao, Y. Wang, R. Xu, C. M. Elliot and D. F. Chen. 1998. The use of visual marker genes as cell specific reporters of *Agrobacterium* mediated T-DNA delivery to wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) Euphytica 99:17-25.

Montoro, P., N. Teinseree, W. Rattana, P. Kongsawadworakul, N. Michaux-Ferriere. 2000. Effect of exogenous calcium on *Agrobacterium tumefaciens* mediated gene transfer in *Hevea brasiliensis* (rubber tree) friable calli. Plant Cell Rep. 19:851-855.

Montoro, P., W. Rattana, V. Pujade-Renaud, N. Michaux-Ferriere, Y. Monkolsook, R. Kanthapura and S. Adunsathapong. 2003. Production of *Hevea brasiliensis* transgenic embryogenic callus lines by *Agrobacterium tumefaciens*: roles of calcium. Plant Cell Rep. 21:1095-1102.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and

bioassays with tobacco tissue cultures. Physiol Plant. 15:473–497.

Nan, G. L., C. S. Tang, A. R. Kuehnle and C. I. Kado. 1997. *Dendrobium* orchids contain an inducer of *Agrobacterium* virulence genes. Physiol. Mol. Plant Pathol. 51:391-399.

Perez Hernandez, J. B., S. Remy, V. Galan Sauco, R. Swennen and L. Sagi 1999. Chemotactic movement and attachment of *Agrobacterium tumefaciens* to banana cells and tissues. J. Plant Physiol. 155:245-250.

Remans, T., P. M. Schenk, J. M. Manners, C. P. C. Grof and A. R. Elliot. 1999. A protocol for the fluorometric quantification of mGFPS-ER and SGFP (S65T) in transgenic plants. Plant Mol. Biol. Rep.17:385-395.

Olhoft, P. M., K. Lin, J. Galbraith, N. C. Nielsen and D. A. Somers. 2001. The role of thiol compounds in increasing *Agrobacterium* mediated transformation of soybean cotyledonary node cells. Plant Cell Rep. 20:731-737.

Olhoft, P. M., L. E. Flaye and D. A. Sowers. 2004. T-DNA locus structure in a large population of soyabean plant transform using the *Agrobacterium* mediated cotyledonary node methods. Plant Biotech. J. 2:289-300.

Opabode, J. T. 2006. *Agrobacterium*-mediated transformation of plants: Emerging factors that influence efficiency. Biotechnol. Mol. Biol. Rev. 3:13-20.

Paz, M. M., H. Shou, Z. Guo, Z. Zhang, A. K. Banarjee and K. Wang. 2004. Assessment of conditions affecting *Agrobacterium* mediated soybean transformation using the cotyledonary node explant. Euphytica 136:167-179.

Sales, M. G., S. H. Park, A. Srivatanakul, and R. H. Smith. 2001. Temperature influence on stable T-DNA integration in plant cells. Plant Cell Rep. 20:701-705.

Shaw, C. H. 1995. *Agrobacterium tumefaciens* chemotaxis protocols. In: K. M. A. Gartland and M. R. Davey (Eds). pp. 29-36. Methods in Molecular Biology: *Agrobacterium* Protocols.

Smith, V. A. and J. Hindley. 1978. Effect of agrocin 84 on attachment of *Agrobacterium tumefaciens* to cultured tobacco cells. Nature 276:498–500.

Sreeramanan, S., M. Maziah, M. P. Abdullah, M. Sariah and R. Xavier. 2006. Transient expression of *gusA* and *gfp* gene in *Agrobacterium*-mediated banana transformation usingtiny meristematic bud. Asian J. Plant Sci. 5:468-480.

Sreeramanan, S., B. Vinod, P. Ranjetta, S. Sasidharan and R. Xavier. 2009. Chemotaxis movement and attachment of *Agrobacterium tumefaciens* to *Phalaenopsis violacea* orchid tissues: An assessment of early factors influencing the efficiency of gene transfer. Trop. Life Sci Res. 20:39–49.

Tao, J. and L. Li. 2006. Genetic transformation of *Torenia fournieri* L. mediated by *Agrobacterium rhizogenes*. South Afr. J. Bot. 72:211-216.

Vergauwe, A., W. Van Geldre, D. Inze, M. Van Montagu and E. Van den Eeckhout. 1998. Factors influencing

Agrobacterium tumefaciens mediated transformation of *Artemisia annua* L. Plant Cell Rep. 18:105-110.

Ying, C., N. Yoshiyuki and S. L. Hu. 2006. Meropenem as an alternative antibiotic agent for suppression of *Agrobacterium* in genetic transformation of orchid. Agric. Sci. China 11:839-846.