Emerging Factors that Influence Efficiency of T-DNA Gene Transfer into *Phalaenopsis Violacea* Orchid via *Agrobacterium Tumefaciens*–mediated Transformation System

Sreeramanan Subramaniam (Corresponding author)

School of Biological Sciences, Universiti Sains Malaysia (USM)

Minden Heights, 11800, Penang, Malaysia

Tel: 60-1-6414-1109   E-mail: sreeramanan@usm.my

Xavier Rathinam

Department of Biotechnology, AIMST University

Batu 3 ½, Jalan Bukit Air Nasi, Bedong, 08100, Kedah, Malaysia

Tel: 604-429-800   E-mail: Xavier@aimst.edu.my

Abstract

An early step in the *Agrobacterium*-mediated transformation of *Phalaenopsis violacea* orchid was investigated in the plant-bacterium interaction. Directed movement in response to chemical attractants is the crucial importance to *Agrobacterium tumefaciens* strains. Chemotaxis of *Agrobacterium tumefaciens* strains (EHA 101 and 105) towards wounded orchid tissues has been studied by using swarm agar plates. The results obtained indicate a minor role of chemotaxis in determining host specificity and suggest that it could not be responsible for the absence of tumorigenesis in *Phalaenopsis violacea* orchid under natural conditions. Various aspects of transformation were examined using transient *gusA* gene expression in efforts to improve the efficiency of producing transformants. Hypervirulent *Agrobacterium tumefaciens* strains, EHA 101 and 105, harboring the pCAMBIA 1304 plasmid containing *gusA* gene as a reporter marker, were used for transformation study. The effects of different concentrations of L-cysteine, silver nitrate, and various temperatures (^{0}C) during co-cultivation in half-strength MS medium supplemented with 5% of banana Mas extract on transformation efficiency were evaluated. Strain EHA 105 proved significantly better than EHA 101 based on higher percentage of *gusA* gene expression and even spread of GUS staining on PLBs. In addition, 200mg.L^{-1} L-cysteine, 60µM silver nitrate, and under the temperature at 24^0C gave the highest percentage of transient GUS expression. The results from transient transformation of PLBs suggested that *Agrobacterium*-mediated transfer of T-DNA is highly efficient. Therefore, by combining the best treatments, an efficient and reproducible *Agrobacterium*-mediated transformation procedure could be continued for the production of transgenic *Phalaenopsis violacea* orchid.

Keywords: *Phalaenopsis violacea*, *Agrobacterium tumefaciens*, Chemotaxis, Transient *gusA* expression

1. Introduction

Phalaenopsis orchid is one of the most important orchids grown for commercial production because of their beautiful flower shape, graceful inflorescence and fragrance. *Phalaenopsis violacea* has the reputation of an almost mystic species and a very pricey rarity. We are focusing on a species closely related to *Phalaenopsis violacea*, known as *Phalaenopsis belina*, a native orchid in Sarawak, Malaysia.

Genetic engineering presents a new approach to the strategy and techniques to transfer potential genes into orchid. *Agrobacterium*-mediated transformation system in orchid plants is preferred because it is a simple technique which does not require expensive equipment; it reduces the copy number of the transgene, potentially leading to fewer problems with transgene co-suppression and instability (Chai et al., 2002; Mishiba et al., 2005). In *Agrobacterium*, chemotaxis plays an important role during the early events of plant–microbe interaction since without it the cell–cell contact, which is required for DNA transfer, could not be established (Mao et al., 2003). The *vir* genes located on Ti-plasmid of *Agrobacterium tumefaciens* are induced in response to chemical signals produced at the plant's wounding site. *Phalaenopsis violacea* orchids are unable to form crown gall tumors after *Agrobacterium* infection *in planta* or *in vitro* excised tissues and therefore chemotaxis of *Agrobacterium* towards

orchid wound exudates has been studied to investigate this first step of interaction. Method for measuring chemotaxis uses swarm agar plates to quantify spatial movement of bacteria (Shaw, 1995).

Early detection of plant transformation events is necessary for the optimization of gene transfer into the orchid genome. Reporter gene, *gus*A (*uid*A) gene encoding for enzyme *β*-glucuronidase (GUS) is used to provide a clear indication of the expression, transient or stable, of transferred genes in transgenic plant cells. Optimization of several factors that influence transient *gus*A gene expression such as the effects of different concentrations of L-cysteine, silver nitrate, and various temperatures (^{0}C) during co-cultivation were evaluated in order to determine the optimum condition of *Agrobacterium*-mediated transfer during the early stages of transformation in *Phalaenopsis violacea*. Protocorm-like bodies (PLBs) are used as a starting material for *Agrobacterium*-mediated transformation, since these contain active undifferentiated cells and could rapidly proliferate under optimum conditions.

The present experiment describes the study of *Agrobacterium* chemotaxis attraction to *Phalaenopsis violacea* cells and tissues swarm agar plates to quantify spatial movement of bacteria. In addition, the optimization of some important aspects of transformation efficiency is therefore, essential to enhance the virulence so as to increase the transformation frequency in *Phalaenopsis violacea* orchid species.

2. Materials and methods

2.1 Plant Materials

Phalaenopsis violacea orchid plants were obtained from Mr. Michael Ooi's orchid nursery in Sungai Dua, Seberang Jaya, Penang (Fig. 1). The *Phalaenopsis violacea* PLBs were obtained from young segments of approximately 1 x 1 cm^2, excised from aseptically raised three-month old *in vitro* seedlings. The PLBs, shoot tip, leaf and root tissues were used for chemotaxis assays. For quantification of *Agrobacterium* attachment experiment, roots, PLBs, shoot tips and calli were used. Protocorm like-bodies (PLBs) of *Phalaenopsis violacea* were obtained from *in vitro* plantlets of three months-old culture using ½ strength Murashige and Skoog (1962) medium supplemented with 5% of Pisang Mas (AA) extract. Cultures were incubated on tissue culture room at 25°C under 16 hours photoperiod with light intensity of 40 μmol.m^{-2}.s^{-1} supplied by white fluorescent tubes. Proliferated PLBs after 2 months were used for the *Agrobacterium*-mediated transformation experiment.

Figure 1

2.2 Agrobacterium tumefaciens strains and culture conditions

Agrobacterium tumefaciens strains, super-virulent strain EHA 101 (pCAMBIA 1304), EHA 105 (pCAMBIA 1304) and control bacteria *E.coli* strain DHα (pMRC 1301) were maintained at -70^{0}C for long term storage in 70% (v/v) glycerol. *Agrobacterium tumefaciens* strains, EHA 101 and EHA 105 contained the disarmed plasmid pCAMBIA 1304 having *hpt11*, β-gluconidase (*gusA*) and green fluorescence protein (*gfp*) gene (Fig. 2). *E. coli* strain DH5α contained plasmid pMRC 1301 with *npt1* and β-gluconidase (*gusA*) genes only. However, this strain was used only as a control bacterium for the quantification of *Agrobacterium* attachment studies. Cultures were grown from single colony in LB medium by incubation at 28^{0}C and 120rpm for 20 hours to reach an optimal density of 0.7 units at 600nm (OD$_{600nm}$). Appropriate antibiotic was included in the medium at the following concentration: kanamycin (50 mgL^{-1}).

Figure 2

2.3 Chemotaxis assays

Chemotaxis assays were carried out according to the modified swarm agar plate method of (Shaw, 1995). Using a toothpick, bacteria were inoculated in the middle of 5 cm diameter Petri dishes containing chemotactic media (CM : 10 mM phosphate buffer, pH 7.0 ; 1 mM ammonium sulfate, 1 mM magnesium sulfate, 0.1 mM potassium-EDTA) partially solidified with 0.2 % (w / v) bacteriological agar. Different types of wounding [excised but otherwise intact PLBs (control: W$_1$), explants injured with scalpel (mild wounding using needle: W$_2$) and (severe wounding using scalpel: W$_3$] were used for this assay. Chemotaxis was quantified after 24 hours incubation at 28^{0}C. The swarming distances from the point of bacterial inoculation towards (T) and backwards (B) the sources of tissue exudates were measured and used to obtain a ratio (R) of the bacterial movement using the following formula:

$$R = T / B$$

Thus, R values over or under 1.00 mean positive or negative chemotaxis, respectively. All experiments were repeated at least four times independently with three replicates each.

2.4 Experimental design: Optimisation of transient gusA gene expression parameters

To assess factors affecting the transformation frequency, three (3) parameters were compared for each factor on transformation efficiency. A range of parameters were evaluated and for each parameter, three (3) replicates were used containing ten (10) PLBs per replicate and were repeated three (3) times. Parameters included different concentrations of L-cysteine (0, 100, 200, 300, 400, 500 and 600 mg.L^{-1}), different concentrations of silver nitrate (0, 20, 40, 60, 80, 100 and 120 μM), and various temperatures (20, 22, 24, 26, 28, 30 and 32^{0}C) during co-cultivation in half-strength MS medium supplemented with 5% of banana, Mas (AA) extract. To determine the optimum conditions for transformation, one factor of the standard conditions was changed each time and the effects on percentage of transient *gusA* gene expression were evaluated.

2.5 GUS histochemical assay

Comparison of the transient expression levels were made by assaying for expression *gusA* gene in the PLBs 3 days after co-cultivation. GUS activity was localized histochemically as described previously by Jefferson (1987). PLBs were immersed in X-Gluc solution (2 mM X-Gluc, 100 mM NaH$_2$PO$_4$ (pH 7.0), 0.5 mM potassium ferricyanide and 50 mM ferrocyanide. The PLBs were then incubated at room temperature (37°C). After staining, the materials were treated with 70% ethanol for 3 days to remove chlorophyll before observation. A sample was scored as transient GUS positive if the 25% blue-region on a PLBs mass was at least 25%.

2.6 Statistical analysis

Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. All analyses were performed at the level 5% using SPSS 10.0 (SPSS Inc. USA).

3. Results and discussion

3.1 Chemotaxis of Agrobacterium tumefaciens

Swarm plates are sloppy agar plates made using a reduced quantity of agar. When inoculated into the center of such plates, the *Agrobacteria* utilize nutrients, thus creating a concentration gradient.

Bacteria incubated on semisolid agar plates swarmed outward from the center point of inoculation, following the gradient created by the presence of diffused chemicals or plant wound exudates at the edges of the plates. Swarming of bacteria was visible to the naked eye and allowed quantitating the chemotactic response of *Agrobacterium tumefaciens*. The chemotactic behavior of *Agrobacterium* was found to be always positive for all the *Phalaenopsis violacea* orchid explants and bacterial strains tested, independently of the wounding level. The overall swarming ratio of the two bacterial strains tested in the presence of orchid tissues ranged between 1.30 and 1.99 indicating a positive effect of the plant exudates on bacterial movement. This effect is shown for the two strains EHA 101 and 105 after assaying with excised tissues tested (Fig. 3). In many cases, bacterial migration to orchid explants accelerated when extra wounding was applied to the tissues and significantly more bacteria were moving as could be seen by the sharper and brighter edge of the swarm For example, the swarming ratios of the strains EHA 101 and 105 in the presence of protocorm-like bodies (PLBs) and shoot tip were always higher with the increased level of wounding (Fig. 3).

However, there were no significant differences ($p < 0.05$) using second (W 2) and third (W 3) level wounding. Whereas for EHA 101, the overall average ratio grouped around 1.55, EHA 105 usually migrated faster towards the explants, which resulted in chemotaxis ratios over 1.75 units. Results obtained from the root and leaf tissues showed a weak positive chemotaxis response suggesting that these explants could be at least partially responsible for the restricted host range of *Agrobacterium* in nature.

Figure 3

Little work has been done to study chemotaxis of *Agrobacterium* to unpurified compound complexes released by intact or wounded plant tissues. Using an agar plate system, Hawes et al. (1988) studied chemotaxis towards root exudates of root cap cells and excised root tips from different plants, observing a strong attraction of motile *Agrobacterium tumefaciens* strains to pea and maize exudates. *VirA* and *VirG* are required for chemotaxis toward phenolic compounds such as acetosyringone (Lee et al., 1996), suggesting a multifunctional role for the *VirA/VirG* system. At a low *vir* inducer concentration, it induces chemotaxis and at a high concentration, it effects *vir* induction (Shaw, 1991). For chemotaxis, *vir* induction does not appear to be required.

3.2 Optimization parameters of gusA transient expression

In order to optimize conditions for *Phalaenopsis violacea* orchid transformation, the effects of several parameters known to influence *Agrobacterium*-mediated DNA transformation were compared. The choice of

Agrobacterium strains played an important role in the transformation process for the efficiency of gene transfer on recalcitrant species of orchid such as *Phalaenopsis violacea* in this study.

Generally, particular strains of *Agrobacterium tumefaciens* will show different levels of competency to the orchid species. The efficiency of strain EHA 101 and 105 has been compared in transforming PLBs of *Phalaenopsis violacea* orchid species. By removing the kanamycin resistance gene from the bacterial chromosome, the *Agrobacterium tumefaciens* EHA 105 strain was created. Several studies have reported that strains EHA 101 and EHA 105 were more effective than other common strains since both are derived from the supervirulent wild-type strain A281 (Hood et al., 1993), whereas strain LBA 4404 was derived from the less virulent strain Ach5 (Hoekema et al., 1983). In our experiments, the transformation efficiency of strain EHA 105 was generally higher than EHA 101 based on the percentage of transient *gusA* gene expression than EHA 101 for all the parameters that were evaluated.

3.3 Different concentrations of L-cysteine

Generally *Agrobacterium tumefaciens* infection caused tissue browning and necrosis on the explants. This is likely to be a common defense response of plants to biotic stress (e.g. pathogen infections) or abiotic stress (e.g. mechanical wounding). The main defense mechanism first activated upon pathogen infection or wounding is oxidative burst (Olhoft et al., 2001). The oxidative burst involves production of reactive oxygen species which are thought to activate programmed cell death (PCD) and to generate a barrier of dead cells around the site of infection (Olhoft et al., 2001).

As a result to increase pathogen defense response and reduce wound response in plants, L-cysteine, an amino acid, not only has the potential to increase the capacity of *Agrobacterium* to infect plant tissues and stably transfer its T-DNA but also to increase the frequency of infected cells that remain viable and become transformed (Olhoft et al., 2001). In addition, cysteine is also an inhibitor of several defense enzymes such as peroxidases (PODs), polyphenol oxidases (PPOs) and enzymatic browning, either directly or indirectly through the action of its thiol group (Olhoft et al., 2001 ; Opabode, 2006).

Therefore, the necessity of L-cysteine during co-cultivation was tested. Five different concentrations of L-cysteine (0, 100, 200, 300, 400 mg.L^{-1}) in the co-cultivation medium were carried out. In this experiment, it was found that 200 mg.L^{-1} L-cysteine resulted in the highest frequency of transient GUS expression of PLBs in both strains, EHA 101 (45%) and 105 (50%). The results demonstrated that L-cysteine generally increased the percentage of transient GUS gene expression in *Phalaenopsis violacea* orchid plants (Fig. 4). However, there were 28 to 36% of transient GUS expression on PLBs observed in the absence of L-cysteine obtained by using both EHA 101 and 105 strains. Therefore, with the addition of L-cysteine in the co-cultivation medium, it was demonstrated to increase *Agrobacterium*-mediated PLBs transformation in *Phalaenopsis violacea*. In addition, it is assumed that L-cysteine and other thiol compounds inhibit wound- and plant pathogen-induced responses, rendering the PLBs explants more susceptible to *Agrobacterium* infection and thereby increasing the capacity of these totipotent plant cells to be transformed. Olhoft and Somers (2001) have successfully increased *Agrobacterium* infection from 37 to 91% of explants in the cotyledonary-node region by amending the solid co-cultivation medium with L-cysteine, which resulted in a five-fold increase in stable T-DNA transfer in newly developed shoot primordial in producing transgenic soybeans (*Glycine max* (L) Merrill). In addition, Zheng et al. (2003) have reported on the optimization of both *Agrobacterium* infection and glufosinate selection in the presence of L-cysteine for the study of expressed sequence tag (EST) and functional genomic analyses (Williams 82) applications in modern genetic analysis and multiplication of soybean (*Glycine max*). Similarly, Enriquez-Obregon et al. (1999) have evaluated the effect of L-cysteine and silver nitrate with known antioxidant activity on the viability of stem section taken from *in vitro* rice plantlets and on their interaction with *Agrobacterium tumefaciens* (AT 2260) containing a shuttle vector bearing the *gusA* and *bar* genes. Olhoft et al. (2001) reported that *Agrobacterium*-mediated transformation of soybean cells and the production of fertile transgenic soybean *Glycine max* (L.) Merrill plants using the cotyledonary-node method were improved by amending the solid co-cultivation medium with L-cysteine.

However, we have observed that browning occurred on PLBs, similar to a hypersensitive response at a higher concentration of L-cysteine, which is more than 200 mg.L^{-1}. Therefore, this would affect the *Agrobacterium* mechanisms of T-DNA transfer initially which will finally reduce the percentage of transient GUS expression. Moreover, it has been reported with a high level of L-cysteine concentration in co-cultivation medium that some negative impacts on *in vitro* plant morphogenesis profiles occured, with 400 mg.L^{-1} L-cysteine exhibited a significant ($p<0.05$) decrease in maize embryos (Frame et al., 2002).

Figure 4

3.4 Different concentrations of silver nitrate (AgNO₃)

Silver nitrate compound is known to inhibit ethylene production, which affects cell division mechanisms. It was reported that in *Agrobacterium*-mediated transformation, ethylene production was increased during *Agrobacterium* infection which resulted in a reduced efficiency of gene transfer mechanism in apple (Seong et al., 2005). In general, it is known with the inclusion of silver nitrate that will suppress the ethylene biosynthesis through Ag^{2+} reducing capacity to bind ethylene receptor produced.

In this study, we examined the effect of silver nitrate in different concentrations on *Phalaenopsis violacea* PLBs for determination of transformation efficiency based on percentage of transient GUS expression. While evaluating the different concentration of silver nitrate in both *Agrobacterium* strains, it was found that the highest percentage of transient GUS expression occurred at 60μM and the lowest was at 120μM in both *Agrobacterium tumefaciens* strains, EHA 101 and EHA 105, respectively (Fig. 5). This could be due to the effect of high concentrations of silver nitrate during co-cultivation is toxic to *Phalaenopsis violacea* PLBs. Inclusion of silver nitrate in co-culture medium has been proven for its anti-ethylene activity which is common with *in vitro* plant cultures. The usage of silver nitrate has shown to have other important effects in plant tissue culture, improving somatic embryogenesis, organogenesis and micropropagation in many species (Zhang et al., 2001).

Addition of silver nitrate in co-culture medium has shown to enhance stable gene transfer in maize (Armstrong and Rout, 2001; Zhao et al., 2001) and Fuji apple (Seong et al., 2005). Silver nitrate significantly suppressed the *Agrobacterium* growth during co-culture without compromising T-DNA delivery and subsequent T-DNA integration. The suppressed *Agrobacterium* growth on the target explants could facilitate plant cell recovery and result in increased efficiency of transformation. Orlikowska (1997) reported that, not only does silver nitrate stimulate direct shoot regeneration from rose (*Rosa indica*) leaves taken from *in vitro* cultures and co-cultivated with *Agrobacterium tumefaciens* for genetic transformation studies, but the compound also inhibits bacterial growth after co-cultivation. They observed that a concentration of 100 mg.L^{-1} silver nitrate after co-cultivation in the plant regeneration medium, retards bacterial growth at least for another 3 weeks. Therefore, addition of silver nitrate is thus able to replace certain commonly used antibiotics such as cefotaxime and carbenicillin, which are often phytotoxic to tissues at least during the first passage after co-cultivation with *Agrobacterium* strains.

Tissue damage by *Agrobacterium tumefaciens* infection has been reported before and seems to be one of the major obstacles for *Agrobacterium*-mediated transformation. Opabode (2006) reported that silver nitrate is an antinecrotic compound which can reduce oxidative burst during the interaction between plant tissue and *Agrobacterium tumefaciens*. In addition, Enriquez-Obregon *et al.* (1999) have evaluated the effect of cysteine and silver nitrate with known antioxidant activity on the viability of stem section taken from *in vitro* rice plantlets and on their interaction with *Agrobacterium tumefaciens* (*AT 2260*) containing a shuttle vector bearing the *gusA* and *bar* genes. Ozden et al. (2004) reported that involvement of ethylene production (enhanced by wounding during explants preparation) leads to browning but can be reduced in the presence of silver nitrate in the medium at optimum levels.

Figure 5

3.5 Temperatures (⁰C)

Temperature has been considered an important factor affecting the efficiency of *Agrobacterium tumefaciens* to transfer the T-DNA to plant cells. There were studies in which temperature was evaluated in a plant-*Agrobacterium tumefaciens* interaction used for GUS transient expression to determine transformation efficiency (Fullner and Nester, 1996 ; Dillen et al., 1997, Sunilkumar and Rathore, 2001).

In order to evaluate whether co-cultivation temperature parameter has effects on *Agrobacterium*-mediated transformation efficacy in *Phalaenopsis violacea*, seven temperature regimes were used to evaluate transformation efficiency, with the results shown in Fig. 6. The highest transformation frequency was observed at 24^0C at which 60-62% PLBs showed GUS activity with no significant difference ($p<0.05$) between the two *Agrobacterium* strains. In tobacco leaves, it was found that a temperature at 24^0C was the co-cultivation temperature regime for transformation, which confirmed the conclusion drawn by Dillen et al. (1997) for T-DNA delivery. The frequencies of percentage of transient GUS positive obtained below and above of temperature 24^0C were decreased markedly (Fig. 6).

However, both Dillen et al. (1997) and Sunilkumar and Rathore (2001) reported that low temperatures below 20^0C during co-cultivation could enhance *Agrobacterium*-mediated efficiency. Similary, Kondo et al. (2000) reported that the ratio of GUS stained calli to the total calli in garlic decreased by 85% at 20^0C and 69% at 24^0C. Fuller et al. (1996) found that low temperatures improved pilus assembly leading to an increased number of pili

on the cell surface due to buffer functioning of the *vir B-vir D4* part of the T-DNA transfer machinery. Fullner and Nester (1996) concluded that temperature did not only affect *vir* A, *vir* B and *vir* D, but the components of the T-DNA complex were sensitive at high temperature. In the present study, the highest efficiency of the PLB transformation in *Phalaenopsis violacea* orchid was obtained at 24^0C among the seven tested temperatures by using *Agrobacterium tumefaciens,* EHA 105 suggesting that appropriately low temperature during co-cultivation can be used for the optimization of transformation. Therefore, it can be concluded that temperature at 24^0C appears to be more optimal for *Agrobacterium* transfer machinery and pilus assembly in *Phalaenopsis violacea* PLBs based on the highest percentage of transient GUS expression on both *Agrobacterium tumefaciens* strains.

Figure 6

4. Conclusions

An understanding of chemotaxis and its methodology is thus important to a full appreciation of *Agrobacterium tumefaciens*-mediated transformation system. It can be concluded that *Agrobacterium* is attracted to exudates from *Phalaenopsis violacea* orchid explants. This suggests that chemotaxis seems to have little or no role in host specificity and consequently does not appear to be a blocking step in *Agrobacterium*–mediated plant transformation. In order to improve *Agrobacterium*-mediated transformation system by obtaining a high level of transient *gusA* expression in *Phalaenopsis violacea* single PLBs, a systematic evaluation of various parameters were carried out. The optimized parameters include the use of *Agrobacterium tumefaciens* strain EHA 105, addition of 200 mg.L^{-1} L-cysteine and, 60 µM silver nitrate. In addition, incubation under the temperature at 24^0C gave the highest percentage of transient GUS expression. The optimized parameters evaluated in this study can be seen as starting points for Agrobacterium-mediated transformation of other recalcitrant *Dendrobium* hybrid plants. The fact that the orchid flower is priced for its aesthetic rather than food value makes it also an ideal system to test-market for GMO acceptance by consumers, by following the statutory procedures for release of GMOs into the environment.

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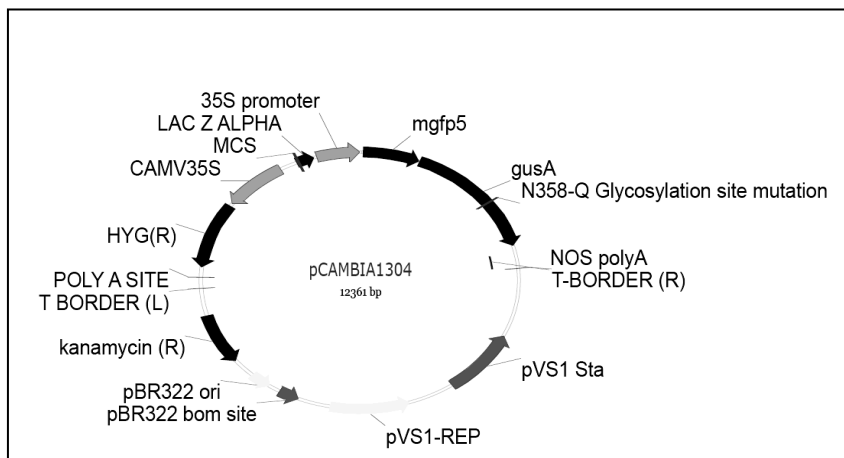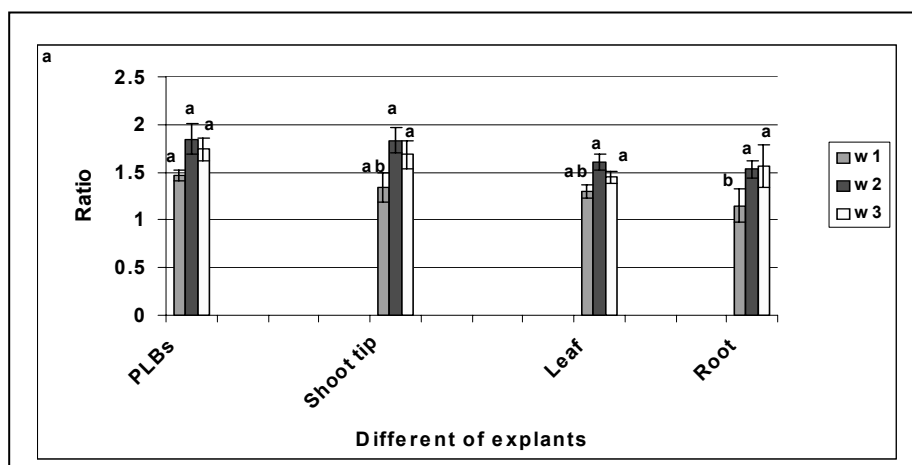Figure 1. The appearance of *Phalaenopsis violacea* orchid plant

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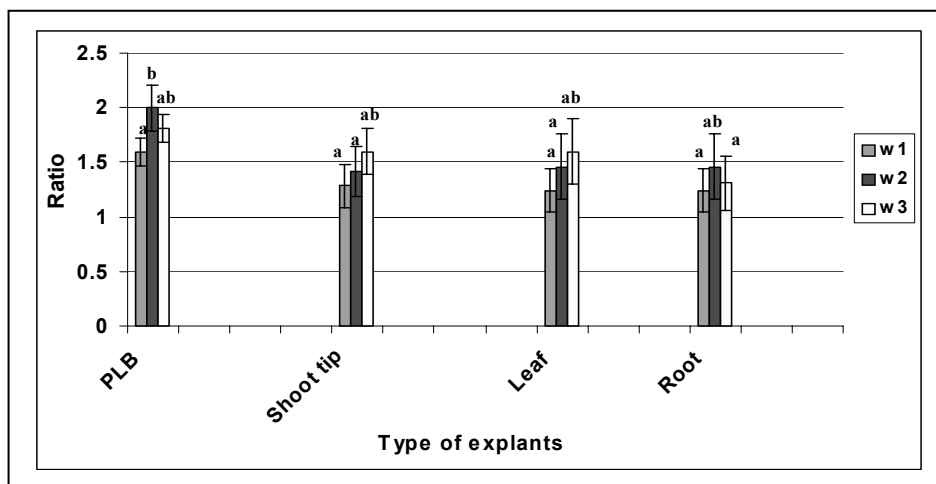
Part A

Part B

Figure 3. Chemotaxis ratios of *Agrobacterium tumefaciens* strains EHA 101 (Panel A) and EHA 105 (Panel B) in the presence of PLBs, shoot tip, leaf and root at low (W1) or increased (W2 and W3) wounding levels. All experiments were repeated for four 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 ($p<0.05$)
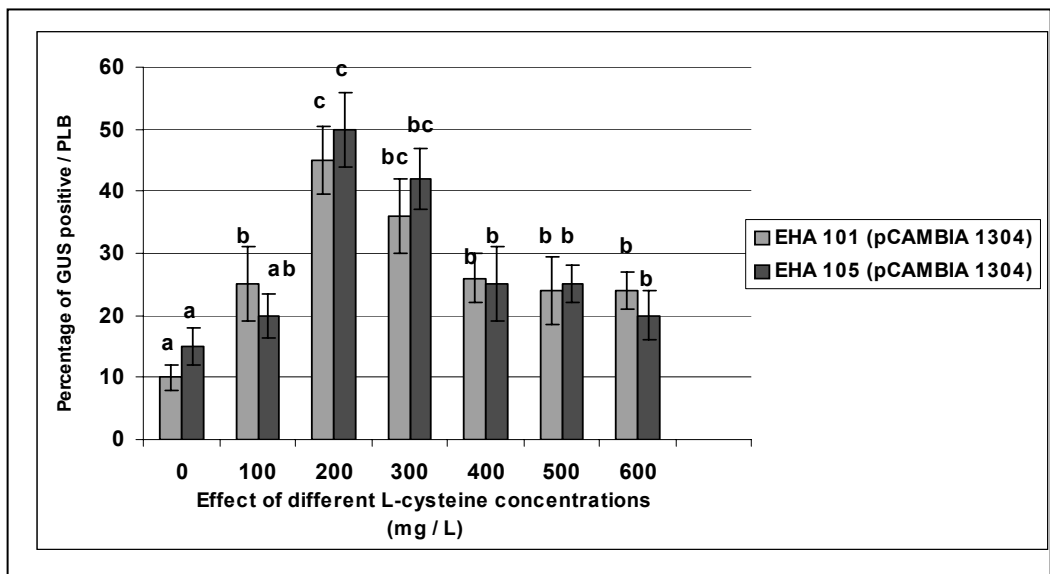
Figure 4. Effect of the L-cysteine treatment on transient *gusA* gene expression in *Phalaenopsis violacea* PLBs. For each parameter, three replicates were used containing ten PLBs per replicate and were repeated three times. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)
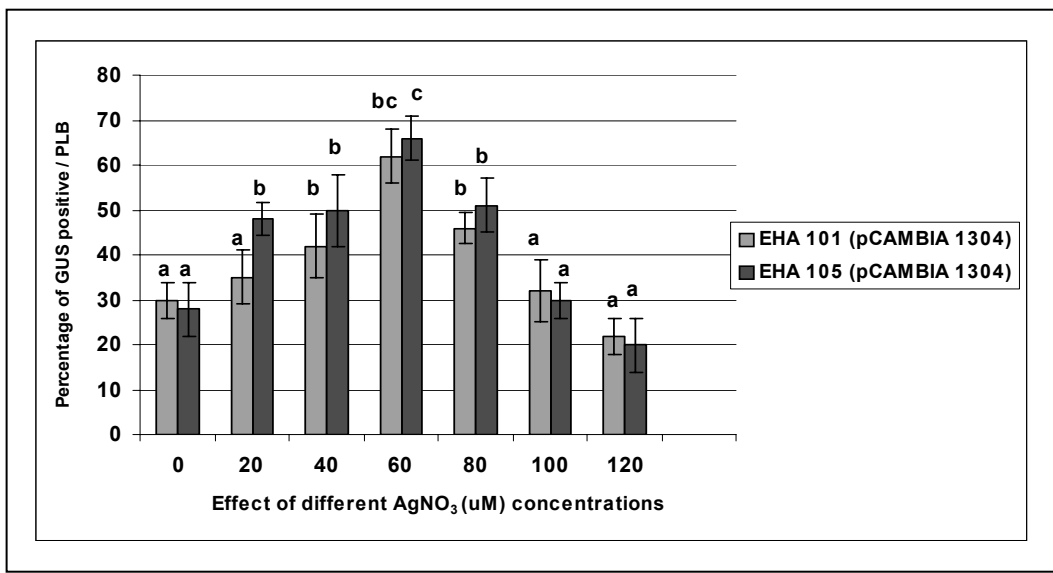

Figure 5. Effect of the silver nitrate ($AgNO_3$) on transient *gusA* gene expression in *Phalaenopsis violacea* PLBs. For each parameter, three replicates were used containing ten PLBs per replicate and were repeated three times. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)
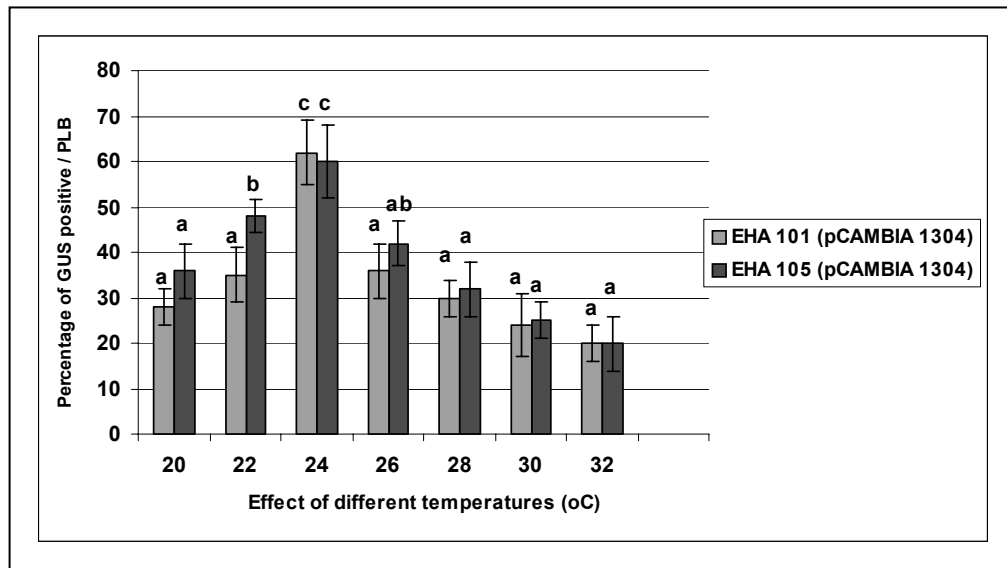
Figure 6. Effect of the different temperatures for gene transfer on transient *gusA* gene expression in *Phalaenopsis violacea* PLBs. For each parameter, three replicates were used containing ten PLBs per replicate and were repeated three times. Data were analyzed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p < 0.05$)