

Chemotaxis Movement and Attachment of *Agrobacterium tumefaciens* to *Phalaenopsis violacea* Orchid Tissues: An Assessment of Early Factors Influencing The Efficiency of Gene Transfer

^{1,2}Sreeramanan Subramaniam*, ¹Vinod Balasubramaniam, ¹Ranjetta Poobathy, ³Sasidharan Sreenivasan and ¹Xavier Rathinam

¹Department of Biotechnology, AIMST University, Batu 3^{1/2}, Jalan Bukit Air Nasi, 08100 Bedong, Kedah, Malaysia

²School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia

³Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia

Abstrak: Peringkat awal transformasi perantaraan *Agrobacterium* di dalam orkid *Phalaenopsis violacea* telah dijalankan bagi menyiasat interaksi diantara tumbuhan dengan bakteria. Pergerakan berarah berdasarkan tindakbalas tarikan kimia merupakan kepentingan utama bagi jenis *Agrobacterium tumefaciens*. Kemotaksis *A. tumefaciens* (EHA 101 dan 105) terhadap tisu orkid yang dilukakan telah dikaji dengan menggunakan *swarm agar plates*. Keputusan yang diperolehi menunjukkan peranan kemotaksis adalah kecil dalam menentukan pengkhususan penerima dan ini menunjukkan ia tidak bertanggungjawab bagi ketiadaan tumoregenesis pada orkid *P. violacea* dalam keadaan semulajadi. Ujian spektrofotometrik GUS dan GFP memberikan informasi mengenai jumlah *A. tumefaciens* yang diinokulasi melekat dengan efektif pada pelbagai jenis tisu orkid. Oleh itu ia boleh disimpulkan bahawa sekurang-kurangnya pada dua peringkat awal interaksi, *A. tumefaciens* didapati serasi dengan *P. violacea* dan ini merupakan asas yang berpotensi bagi pembangunan genetik transformasi.

Kata kunci: *Phalaenopsis violacea*, *Agrobacterium tumefaciens*, Kemotaksis, Pelekatan Bakteria

Abstract: An early step in the *Agrobacterium*-mediated transformation of *Phalaenopsis violacea* orchid was investigated to elucidate the plant-bacterium interaction. Directed movement in response to chemical attractants is of crucial importance to *Agrobacterium tumefaciens* strains. Chemotaxis of *A. 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 for chemotaxis in determining host specificity and suggest that it could not be responsible for the absence of tumourigenesis in *P. violacea* orchid under natural conditions. The spectrometric GUS and green fluorescent protein (GFP) assays provided information on the amount of inoculated *A. tumefaciens* that effectively bound to various orchid tissues. It can be concluded that, at least during the two early steps of interaction, *A. tumefaciens* appears to be compatible with *P. violacea*, indicating a potential basis for genetic transformation.

Keywords: *Phalaenopsis violacea*, *Agrobacterium tumefaciens*, Chemotaxis, Bacterial Attachment

*Corresponding author: sreeramanan@usm.my

INTRODUCTION

Phalaenopsis orchid is one of the most important orchids grown for commercial production because of its beautiful flower shape, graceful inflorescence and fragrance. Among orchid lovers, *P. violacea* has the reputation of an almost mystic species and a very expensive rarity.

Genetic engineering presents a new approach to the strategy and techniques used to transfer potential genes into orchids (Liau *et al.* 2003; Chin *et al.* 2007). The *Agrobacterium*-mediated transformation system is preferred for use in orchid plants because it is a simple technique that does not require expensive equipment; it reduces the copy number of the transgene, potentially leading to fewer problems with transgene co-suppression and instability (Cheng *et al.* 1997; Hiei *et al.* 1997; Liau *et al.* 2003; Chin *et al.* 2007). Bacterial chemotaxis is considered to be the first step in the interaction between *Agrobacterium* and plant cells during the process of bacterial infection. *A. tumefaciens* is attracted to a variety of sugars, typically of plant exudates. A response highly sensitive to wound exudates may help to guide *A. tumefaciens* directly to wounded plant cells (Shaw 1991). *P. violacea* orchid is unable to form crown gall tumours after *Agrobacterium* infection *in planta* or in excised tissues *in vitro*. Therefore chemotaxis of *Agrobacterium* towards orchid wound exudates has been studied to investigate this first step of interaction.

Bacterial chemotaxis involves the movement of motile cells towards or away from chemicals in response to a gradient of attractant or repellent, respectively (Mao *et al.* 2003). 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. The *vir* genes located on the Ti-plasmid of *A. tumefaciens* are induced in response to chemical signals produced at the plant wound site. These signals include low pH, phenolic compounds, and monosaccharide components of the plant cell wall. A chemotaxis cluster has been described in a region of the *Agrobacterium* chromosome that resembles chemotaxis operons identified in other members of the same group of proteobacteria (Wright *et al.* 1998).

The preferred methodology for measuring chemotaxis uses swarm agar plates to quantify spatial movement of bacteria (Shaw 1995). When inoculated onto the centre of semisolid agar plates, bacteria migrate, following a concentration gradient of compounds to which they are tactically responsive. This gradient could be directly created by swarming bacteria while consuming nutrients present in the medium. The gradient could also be the result of compounds from an external source placed at some distance from the point where bacteria are inoculated (Mao *et al.* 2003). After a period of incubation, the distance covered by swarming bacteria provides an indication of the chemotactic response to tested compounds.

Following chemotaxis, a second early step in the process of infection is the attachment of *Agrobacterium* to the plant cell. Binding of *A. tumefaciens* to target plant cells is essential for tumourigenesis and appears to be mediated by specific receptors located on the bacterial and plant cell surfaces. *A. tumefaciens* binds to the plant cell in a two-step process, in which an initial loose attachment

of individual bacterial cells is followed by tight binding and massive aggregation of bacteria at the host cell surface (Perez Hernandez *et al.* 1999).

It has been shown that *Agrobacterium*, exhibiting some common characteristics, is able to attach to cells of a wide range of plant species (Kado 1998; Vergauwe *et al.* 1998). Though attachment of *Agrobacterium* to plant cells can be observed through a number of microscopy techniques, the specificity of the cell-cell contact is demonstrated most accurately by a quantitative measurement of the binding capacities of attachment-competent bacteria. 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. The present experiment describes the study of *Agrobacterium* attachment to *P. violacea* cells and tissues, quantifying bacterial attachment through spectrophotometric GUS and GFP expression assays.

MATERIALS AND METHODS

Plant Materials

The *P. violacea* orchid plants were obtained from Mr. Michael Ooi's orchid nursery in Sungai Dua, Seberang Jaya, Penang (Fig. 1). The *P. violacea* protocorm-like bodies (PLBs) were obtained from young segments of approximately 1 x 1 cm², 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, roots, PLBs, shoot tips and calli were used. PLBs of *P. violacea* were obtained from *in vitro* plantlets which had been cultured for three months using ½ strength Murashige and Skoog (1962) medium supplemented with 5% Pisang Mas (AA) extract. Cultures were incubated in a tissue culture room at 25°C under 16-hour photoperiod with light intensity of 40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ supplied by white fluorescent tubes. After two months, proliferated PLBs were used for the *Agrobacterium*-mediated transformation experiment.



Figure 1: The appearance of *P. violacea* orchid plant.

A. *tumefaciens* Strains and Culture Conditions

A. tumefaciens strains, super-virulent strain EHA 101 (pCAMBIA 1304), EHA 105 (PCAMBIA 1304) and *Escherichia coli* strain DH α (pMRC 1301) (control bacteria) were maintained at -70°C for long-term storage in 70% (v/v) glycerol. *A. tumefaciens* strains EHA 101 and EHA 105 contained the disarmed plasmid pCAMBIA 1304 which contains genes for hygromycin (*hyg*), β -glucuronidase (*gusA*) and *gfp* (Fig. 2). The DH α *E.coli* strain contained pMRC 1301 plasmid with *npt11* and β -glucuronidase (*gusA*) genes only. This latter strain was used as a control in the *Agrobacterium* attachment studies. Cultures were grown from a single colony in LB medium by incubation at 28°C and 120rpm for 20 hours to reach an optimal density of 0.7 units at 600nm ($\text{OD}_{600\text{nm}}$). Appropriate antibiotic was included in the medium (kanamycin, 50 mgL^{-1}).

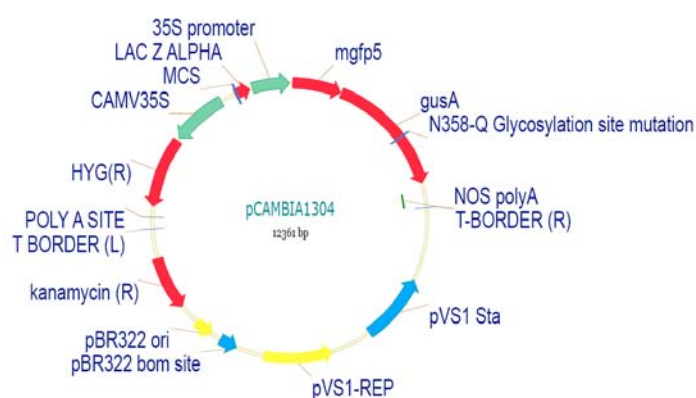


Figure 2: Schematic diagram of the pCAMBIA 1304 plasmid. The binary vector pCAMBIA 1304 (CSIRO, Australia) harbouring the reporter *gusA* and *mgfp* genes driven by the *CaMV* 35S promoter.

Chemotaxis Assays

Chemotaxis assays were carried out according to Shaw's protocol (1995) for the modified swarm agar plate method. Using a toothpick, bacteria were inoculated in the middle of a Petri dish (5 cm diameter) containing chemotactic media (CM: 10mM phosphate buffer, pH 7.0; 1mM ammonium sulfate; 1mM magnesium sulfate; 0.1mM potassium-EDTA) partially solidified with 0.2% (w/v) bacteriological agar. Several types of wounding were used for this assay: 1) excised but otherwise intact PLBs (control: w1); 2) explants subjected to mild wounding using a needle: w2); 3) explants subjected to severe wounding using a scalpel: w3). Chemotaxis was quantified after 24 hour incubation at 28°C . The swarming distances from the point of bacterial inoculation towards (T) and backwards (B) from 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 represent positive or negative chemotaxis, respectively. All experiments were repeated at least four times independently, with three replicates each.

Quantification of Bacterial Attachment to *P. violacea* (PLBs), Shoot Tip, Leaf and Root Explants Through Spectrophotometric Measurement of GUS and GFP Expression in Genetically Marked *A. tumefaciens* Strains

Bacterial attachment assays were carried out according to the methodology of Perez Hernandez *et al.* (1999). PLBs (3–4mm), shoot tips, and leaf and root explants were prepared. During preparation, explants were maintained in 25 mM phosphate buffer (pH 7.5). To confirm the binding capacity of *Agrobacterium tumefaciens* EHA 101 and 105, the *Escherichia coli* strain DH α was included in all experiments as a negative control. For infection, 1.5 ml Eppendorf tubes filled with 1 ml of buffer were loaded with 50 μ L aliquots of buffer-suspended bacteria. Tubes were then incubated in a rotary shaker at 28°C at 25 rpm for 2 hours. After this period, unbound bacteria were removed by washing the explants twice with 1 ml fresh buffer. Samples were vortexed for 30 seconds in order to discard unattached bacteria. β -glucuronidase activity in the samples was measured with a modification of the spectrophotometric assay described by Wilson *et al.* (1992). Washed explants were transferred to 1 ml of extraction buffer [50 mM sodium phosphate (pH 7.0), 10 mM dithiothreitol, 1 mM sodium EDTA, 0.1% (v/v) sodium lauryl sarcosine, 0.1% (v/v) Triton X-100], vortexed and incubated at 37°C for 10 minutes. The GUS enzyme substrate p-nitrophenyl β -D-glucuronide was added at a final concentration of 1 mM; after incubation at 37°C for 30 minutes, reactions were stopped by the addition of 400 μ L of a 400 mM Na₂CO₃ solution. GUS activity was quantified by measuring light absorbance at 415 nm (A₄₁₅). Absorbance was also measured from explants containing uninfected tubes in order to determine the levels of light absorption at 415 nm from plant-released compounds. We also measured the absorbance from inoculated tubes in the absence of plant material in order to determine total enzymatic activity in the inoculum. GFP activity in the samples was measured as outlined for the quantification of β -glucuronidase activity, except no substrate was used and the activity was quantified by measuring direct light absorbance at 510 nm (A₅₁₀), 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 explant type and bacterial strain.

Statistical Analysis

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

RESULTS AND DISCUSSION

Chemotaxis of *A. tumefaciens*

Swarm plates are sloppy agar plates made using a reduced quantity of agar. When inoculated into the centre of such plates, the *Agrobacterium* utilize nutrients, thus creating a concentration gradient. Bacteria respond by steadily moving outward, consuming nutrients and creating a gradient as they go. Swarm plates have a number of applications (Shaw 1995):

- Routinely, cultures for chemotaxis assays are enriched for highly motile populations by three passages through swarm plates, taking bacteria from the outermost edge at each passage.
- Spontaneous mutants can be selected by taking *Agrobacterium* from the centre of a swarm.
- By incorporating potential attractants into minimal swarm plates, it is possible to perform crude tests of their attractiveness. This does usually require that the attractant can also be metabolized.

In addition to a swarm agar plate system, the capillary assay described by Perez Hernandez *et al.* (1999) was tested for studying chemotactic movements of *Agrobacterium*. Although reported to be highly sensitive, capillary assays are in general difficult to set up and large numbers of replicates are required to obtain reliable results (Shaw 1995). Moreover, *Agrobacterium* is characterized by the production of extracellular polysaccharides which, especially in certain strains such as EHA 101 and 105, result in the aggregation of bacterial cells in culture. This makes it difficult to accurately quantify bacteria in a sample through determination of colony forming units on a plate. Therefore, the swarm agar plate method was chosen for the study of chemotaxis.

Bacteria incubated on semisolid agar plates swarmed outward from the central point of inoculation, following the gradient created by the presence of diffused chemicals or plant wound exudates at the edges of the plates. Bacterial swarming was visible to the naked eye and allowed us to quantify the chemotactic response of *A. tumefaciens*. The chemotactic behaviour of *Agrobacterium* was found to be invariably positive for all the *P. 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 demonstrated by the results

obtained from the two strains EHA 101 and 105 after assaying the excised tissues (Fig. 3). In many cases, bacterial migration to orchid explants accelerated when extra wounding was applied to the tissues; 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 PLBs and shoot tip were always higher with an increased level of wounding (Fig. 3).

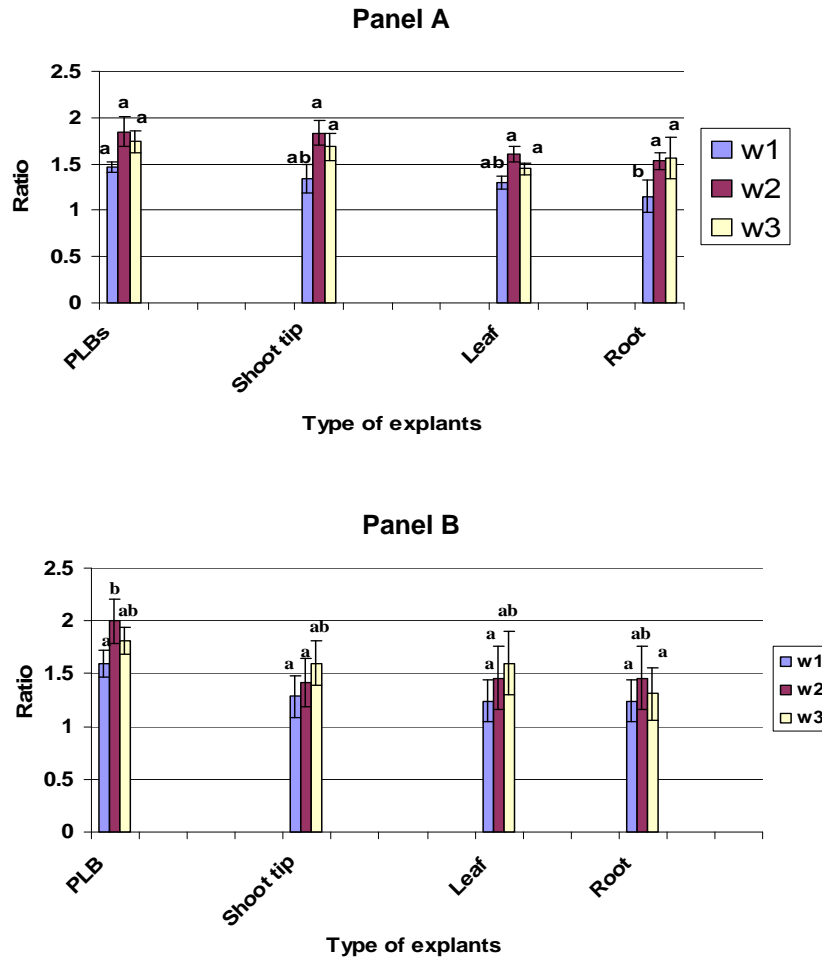


Figure 3: Chemotaxis ratios of *A. tumefaciens* strains EHA 101 (Panel A) and EHA 105 (Panel B) in the presence of PLBs, shoot tip, leaf and root at low (w1) or increased (w2 and w3) wounding levels. All experiments were repeated four times. Data were analyzed using one-way ANOVA and the differences were contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p < 0.05$).

However, there was no significant difference using second- (w2) and third-level (w3) wounding. For EHA 101, the overall average ratio grouped around 1.55, while 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.

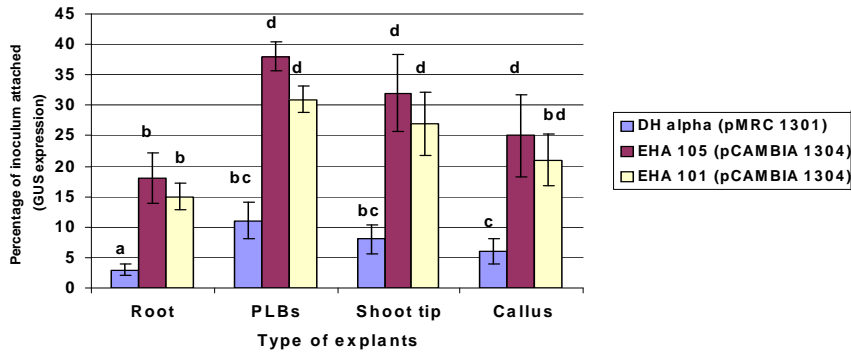
Few studies have examined chemotaxis of *Agrobacterium* to unpurified compound complexes released by intact or wounded plant tissues. Using an agar plate system, Hawes *et al.* (1988) studied chemotaxis toward the root exudates of root cap cells and excised root tips from different plants, observing a strong attraction of motile *A. 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, the compound induces chemotaxis; at a high concentration, it affects *vir* induction (Shaw 1991). *vir* induction does not appear to be required for chemotaxis.

Quantification of Bacterial Attachment

Aside from the chemotaxis assay, a system for the quantification of bacterial attachment was developed, which provided information about (i) the specificity of the process for attachment-competent *Agrobacterium* and (ii) the amount of inoculated bacteria that effectively bound to plant cells. Microscopic examination of bacteria interacting with the plant cells indicated a significant propensity to attach in a polar fashion (Smith & Hindley 1978). Quantitative estimation of binding by *Agrobacterium* to plant cells has revealed two types of interactions: 1) a nonspecific, non-saturable, aggregation-like interaction readily removed via washes with a buffered salt solution; 2) 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 determine whether the same pattern characterizes the *Agrobacterium*-banana interaction, before attempting transformation in this species. The spectrophotometric GUS and GFP assays used for quantification of bacterial attachment revealed the increased binding ability of the attachment-efficient *Agrobacterium* strains (EHA 101 and 105) as compared to the DH α strain of naturally non-attaching *E. coli* (Fig. 4).

Whereas no major differences were observed among the two *Agrobacterium* strains tested, attachment to protocorm-like bodies showed the greatest differences when compared to attachment among the binding-deficient *E. coli* bacteria (control bacteria), using GUS expression (Fig. 4; Panel A). This may be because protocorm-like body segments contain the highest exposed cell surface of all explants tested and thus provide the most numerous binding sites for effective attachment of competent bacteria. In other types of explant, the proportion of intercellular spaces where bacteria could escape from vigorous washings increased with respect to the sites available for effective binding. This diminished the differences between binding-deficient bacteria and bacteria that effectively colonise these tissues. Both GUS and GFP expression revealed that the highest percentage of inoculum attached in PLB explants (Fig. 4).

Panel A



Panel B

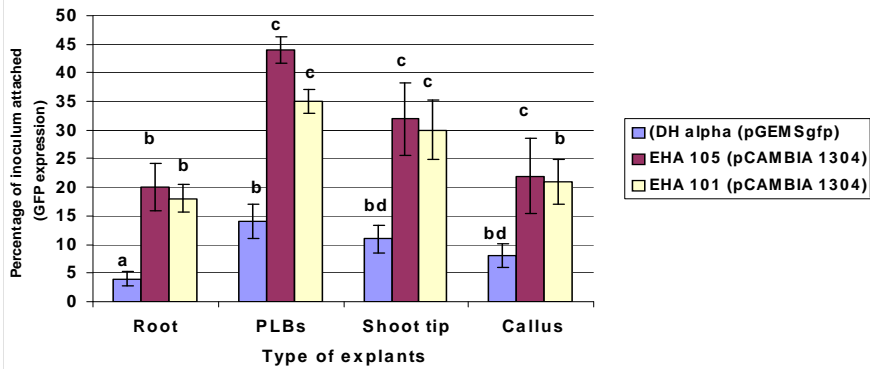


Figure 4 : Quantification of bacterial attachment to *P. violacea* PLBs, shoot tip, leaf and root explants through spectrophotometric measurement of GUS (Panel A) and GFP (Panel B) expression in genetically tagged bacteria. Values correspond to the percentages of inoculated bacteria remaining attached to cells after extensive washing of infected tissues. Data were analysed using one-way ANOVA. The differences were contrasted using Duncan’s multiple range test. Different letters indicate values are significantly different ($p < 0.05$)

CONCLUSION

An understanding of chemotaxis and its methodology is important to a full appreciation of the *A. tumefaciens*-mediated transformation system. It can be concluded that *Agrobacterium* is attracted to exudates from *P. 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. Under natural conditions, chemotaxis of *Agrobacterium* to wounded plant cells is the first event required for bacterial infection and disease development, a process of primary importance for an opportunistic pathogen. Therefore, the absence of directed bacterial movement in the presence of wounded *P. violacea* explants could at least partially explain why this plant species does not naturally form tumours. By means of bacterial swarming, it was observed that *Agrobacterium* was attracted to wound exudates from different types of explants. The quantification system proved that *Agrobacterium* is able to attach specifically to different types of *P. violacea* orchid cells, as revealed by the GUS and GFP spectrometric assays. Once *Agrobacterium* is in close proximity to wounded tissues, the next step required for the development of plant tumours involves attachment to plant cells. *Agrobacterium* was able to bind to wounded as well as to unwounded plant cell surfaces, challenging controversial reports of the necessity of plant cell damage for transformation (at least during the initial phase of bacterial colonization). In addition, the binding process was quantified, which provided further evidence for the specific ability of virulent *Agrobacterium* to colonize tissues from PLBs of *P. violacea*.

ACKNOWLEDGEMENT

The authors wish to thank Dr. Richard I.S. Bretell from CSIRO, Australia for the pCAMBIA 1304 plasmid. This research was supported financially by the Malaysian Science and Technology Toray Foundation (2005).

REFERENCES

- Cheng M, Fry J E, Pang S, Zhou H, Hironaka C M, Duncan D R, Conner T W and Wan Y. (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiology* 115: 971–980.
- Chin D P, Mishiba K and Mii M. (2007). *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cymbidium*. *Plant Cell Reports* 26: 735–743.
- Gurlitz R H G, Lamb P W and Matthyse A G. (1987). Involvement of carrot surface proteins in attachment of *Agrobacterium tumefaciens*. *Plant Physiology* 83: 564–568.
- Hawes M C, Smith L Y and Howarth A J. (1988). *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. *Molecular Plant-Microbe Interactions* 1: 182–186.
- Hiei Y, Komari T and Kubo T. (1997). Transformation of rice mediated by *Agrobacterium tumefaciens*. *Plant Molecular Biology* 35: 205–218.

- Kado C I. (1998). *Agrobacterium*-mediated horizontal gene transfer. *Genetic Engineering* 20: 1–24.
- Lee Y W, Jin S G, Sim W S and Nester E W. (1996). The sensing of plant signal molecules by *Agrobacterium*: Genetic evidence for direct recognition of phenolic inducers by the *VirA* protein. *Gene* 179: 83–88.
- Liau C H, You S J, Prasad V, Hsiao H H, Lu J C, Yang N S and Chan M T. (2003). *Agrobacterium tumefaciens*-mediated transformation of an *Oncidium* orchid. *Plant Cell Reports* 21: 993–998.
- Mao H, Cremer P S and Manson M D. (2003). A sensitive, versatile microfluidic assay for bacterial chemotaxis. *Proceedings of the National Academy of Sciences of the United States of America*. 100(9): 5449–5454.
- Murashige T and Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologica Plantarum* 15: 473–497.
- Perez Hernandez J B, Remy S, Galan Sauco V, Swennen R and Sagi L. (1999). Chemotactic movement and attachment of *Agrobacterium tumefaciens* to banana cells and tissues. *Journal of Plant Physiology* 155: 245–250.
- Remans T, Schenk P M, Manners J M, Grof C P L and Elliot A R. (1999). A protocol for the fluorometric quantification of mGFP5-ER and sGFP (S65T) in transgenic plants. *Plant Molecular Biology Reporter* 17: 385–395.
- Shaw C H. (1991). Swimming against the tide: Chemotaxis in *Agrobacterium*. *BioEssays* 13: 25–29.
- Shaw C H. (1995). *Agrobacterium tumefaciens* chemotaxis protocols. In K M A Gartland and M R Davey (eds.). *Agrobacterium Protocols: Methods in molecular biology*. Totowa, NJ: Humana Press Inc., 44: 29–36.
- Smith V A and Hindley J. (1978). Effect of agrocin 84 on attachment of *Agrobacterium tumefaciens* to cultured tobacco cells. *Nature* 276: 498–500.
- Vergauwe A, Van Geldre E, Inze D, Van Montagu M and Van den Eeckhout E. (1998). Factors influencing *Agrobacterium tumefaciens*-mediated transformation of *Artemisia annua* L. *Plant Cell Reports* 18: 105–110.
- Wilson K J, Hughes S G and Jefferson R A. (1992). The *Escherichia coli gus* operon: Induction and expression of the *gus* operon in *E. coli* and the occurrence and use of GUS in other bacteria. In S R Gallagher (ed.). *GUS protocols: Using the GUS gene as a reporter of gene expression*. San Diego: Academy Press Inc., 7–22.
- Wright E L, Deakin W J and Shaw C H. (1998). A chemotaxis cluster from *Agrobacterium tumefaciens*. *Gene* 220: 83–89.