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Research Note

Chemotactic movement and bacterial attachment of *Agrobacterium tumefaciens* towards protocorm-like bodies (PLBs) of *Dendrobium* sonia-28

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Abstract

Dendrobium orchids are economically important as cut flowers. The transformation of *Dendrobium* using *Agrobacterium tumefaciens* has emerged as an important protocol in improving the quality of the orchid. The T-DNA gene transfer conditions have to be optimised to ensure successful *Agrobacterium* transformation. *Agrobacterium* chemotaxis and attachment studies were carried out on protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 to determine the potential strength of the *Agrobacterium*-PLB interaction during the transformation event. The chemotactic response and attachment of the *Agrobacterium* to the PLBs were positive. The greatest bacterial movement (ratio between 1.2-1.5) was obtained when 3-4mm PLBs were used for transformation, when compared to 1-2mm PLBs. Higher *Agrobacterium* attachment rates (40-75%) were obtained in 3-4mm PLBs when compared to 1-2mm PLBs. Protocorm-like body-wounding does not significantly attract the bacteria, but efficiently increased bacterial attachment sites on the PLBs. The results concluded that the chemotactic response and attachment of *Agrobacterium* were positive to the PLBs of *Dendrobium* sonia-28, indicating a potential for genetic transformation.

Keywords: Bacterial attachment; Chemotaxis; *Dendrobium* sonia-28; PLBs.
Abbreviations: PLBs-Protocorm-like bodies; BAP-6-benzylaminopurine; LB-Luria Bertani; SEM-Scanning electron microscope.

Introduction

Dendrobium orchids are in high demand in the cut flower industry (Sarntinoranont and Wannakrairoj, 2010). Genetic engineering *via* *Agrobacterium*-mediated transformation offers a promising avenue in the development of specific orchid traits due to cost and time efficiency (Teixeira da Silva et al., 2011). *Agrobacterium*-mediated transformation takes advantage of the natural infection mechanism of *Agrobacterium tumefaciens* towards wounded plants tissue, resulting in a neoplastic growth disease known as the crown gall (Stafford, 2000; Teixeira da Silva et al., 2011). In nature, *Agrobacterium* first recognises and senses a wounded host cell, and then moves towards the wounded tissue (Matthysse, 2006; Citovsky et al., 2007). The *Agrobacterium* capability to interact with the host is highly influenced by their motility. Deakin et al. (1999) demonstrated that detrimental effect to the flagellar cluster formation which facilitates motility may cost the virulence in *Agrobacterium*. Merritt et al. (2007) demonstrated that *A. tumefaciens* ability for surface attachment is highly affected by their motility. Therefore, it is crucial to determine the *Agrobacterium* behaviour and motility before attempting any transformation process. Chemotaxis assay using a simple swarm plate agar protocol offers a method to measure the bacteria-plant interaction and the bacterial motility (Sreeramanan et al., 2009). To initiate infection, the *Agrobacterium* must attach efficiently to the host plant. Orchids, like other monocots, are not natural hosts for *Agrobacterium*. The determination of *Agrobacterium* attachment on plant cell is therefore important to verify *Agrobacterium* interaction on monocot cells. Verma et al. (2008) and Sreeramanan et al. (2009) showed that *Agrobacterium* attachments were achieved on the callus of sorghum and the PLBs of *Phalaenopsis* orchid. *Agrobacterium* attachment can be studied through both microscopy and spectrophotometer quantification of GUS or GFP expressions to determine the number of inoculated bacteria which attached efficiently to the plant (Perez Hernandes, 2000). This preliminary study evaluates the interaction between *Agrobacterium tumefaciens* strain LBA 4404 and PLBs of *Dendrobium* sonia-28 as an early assessment for plant-bacterial interaction.

Results and discussion

Bacterial chemotaxis

In the first 24 hours (Fig 1), the bacterial chemotaxis accelerated towards 3-4mm PLBs at a ratio ranging from 1.2-1.5 units, showing high bacteria motility crucial in determining bacterial virulence in facilitating gene transfer. Low chemotactic response (<1.3 units) was observed in 1-2mm PLBs. No significant differences were detected between the wounding conditions applied on both PLBs sizes. However, unwounded PLBs showed higher bacterial chemotaxis compared to wounded PLBs in both sizes (1-2mm=1.05 units; 3-4mm=1.25 units). After 48 hours of incubation (Fig 1), the bacterial motility decreased. The chemotactic ratios were recorded at 1.1-1.25 units in 3-4mm PLBs, and 0.9-1.1 units in 1-2mm PLBs. No significant

differences were detected in the chemotactic movements of the *Agrobacterium* when different types of wounding were applied on different-sized PLBs. However, the greatest chemotactic movement was recorded when large and wounded PLBs (1.25 units) were used. The overall chemotactic response of *Agrobacterium* LBA 4404 was positive to the PLBs of *Dendrobium* sonia-28, with the exception of the small and wounded PLBs. From all the bacterial growth movements observed in this study (Fig 3a-d), the sharpest edge of the bacterial swarm was observed in the plate containing large and wounded PLBs (Fig 3d). The first vital step in the bacterial infection process is the attraction of the bacteria towards the target explant. The swarm agar plate offers a convenient protocol for chemotactic analyses. The bacteria are inoculated in the centre of the plate and the tested chemical compound or plant cells are placed at a distance from the point of inoculation (Perez Hernandes et al., 1999). The bacteria then consume nutrients from the media and slowly migrate outward from the point of inoculation, creating a concentration gradient (Shaw, 1995; Lengeler, 2004). The distance covered by the swarming bacteria is then measured to determine the chemotactic response of the bacteria towards the tested chemical or tissue exudate (Perez Hernandes, 2000).*Agrobacterium tumefaciens* chemotaxis is a response from the bacteria towards phenolic compounds released from plant wounds, which mediates the phosphorylation of the virulent genes *virA* and *virG* (Shaw, 1993; Wright et al., 1998). Nan et al. (1997) reported that the presence of both aryl β-glycoside and coniferyl alcohol in the PLBs of *Dendrobium* is essential in inducing the virulence genes of the bacteria. This study however indicates no significant difference between the two wounding conditions, suggesting that wounding may not be necessary in inducing an interaction between *Agrobacterium* and *Dendrobium* sonia-28. The PLBs may have released chemotactic attractants that bypassed the need of wounding to induce bacterial virulence. Brencic et al. (2005) showed that unwounded tobacco seedling were able to release phenolic compounds that induced the virulence genes of *Agrobacterium*.

Bacterial attachment

Agrobacterium attachment is significantly higher in 3-4mm PLBs ($p<0.05$), producing between 40-75% GUS expression compared to 1-2mm PLBs, which produced less than 20% GUS expression (Fig 2). *Escherichia coli*, a bacterium naturally non-pathogenic to the PLBs, displayed less than 10% to completely negative GUS expressions. Protocorm-like body wounding increased the bacterial attachment percentages in both size ranges. Large wounded PLBs displayed 20% GUS expression, while no significant differences were detected in the percentage of GUS expression for different types of wounding in 1-2mm PLBs. Microscopy observations showed that fibril networks glued the bacteria to one another and anchored them to the plant surface (Fig 3d). The bacterial attachment process involves two essential steps: firstly, bacterial attachment to the plant cell wall, and secondly, the firm and irreversible attachment mediated by bacterial appendages or other binding mechanisms. Smith et al. (1992) showed that the *Rhizobium* attachment to plant roots involved an initial direct attachment of bacteria to the root hairs mediated by the calcium-binding proteins of the bacteria, followed by a firm attachment mediated by bacterial appendages and/or the plant lectins. Gorski et al. (2003) suggested that the ability of bacteria to adhere strongly to plant cells, even withstanding washing of

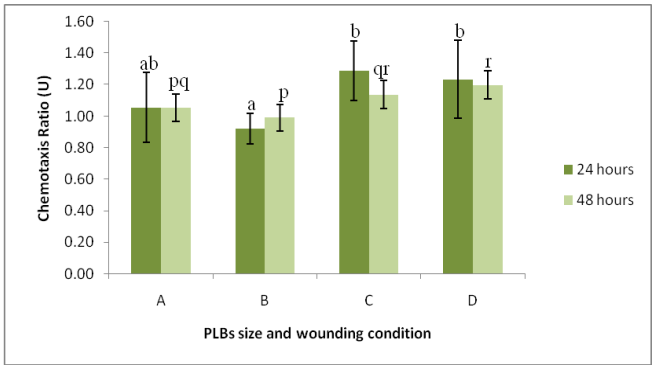

Fig 1. Chemotaxis of *A. tumefaciens* strain LBA4404 towards different sizes and wounding types of PLBs after 24 hours and 48 hours. A=small, unwounded; B=small, wounded; C=large, unwounded; and D= large, wounded. Different letters represent significant differences at the 5% level.

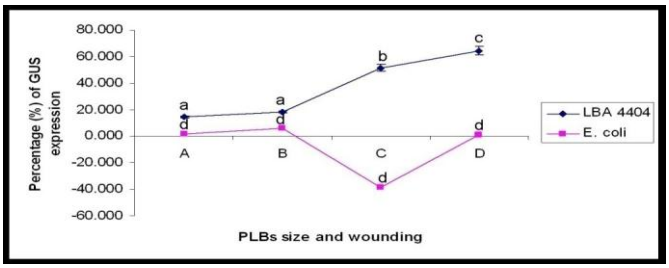

Fig 2. Percentage of *A. tumefaciens* strain LBA4404 and *E. coli* attachment towards different sizes and wounding types of PLBs. A=small, unwounded; B=small, wounded; C=large, unwounded; and D=large, wounded. Different letters represent significant differences at the 5% level.

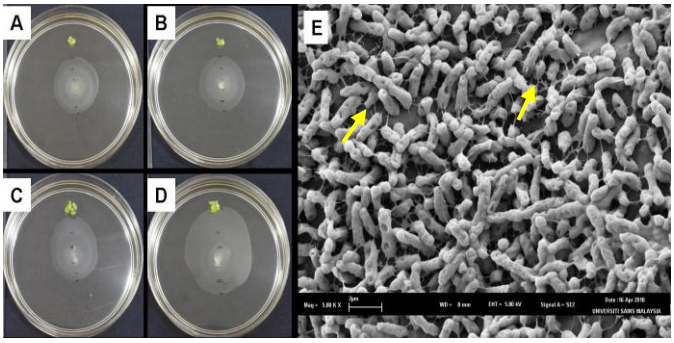

Fig 3. (A-D) Chemotaxis movement of *A. tumefaciens* in response to different-sized PLBs and wounding types. A=small, unwounded; B=small, wounded; C=large, unwounded; and D=large, wounded. The chemotaxis of *Agrobacterium* was the fastest in large PLBs compared to the small PLBs, with no difference observed in wounding types. (E) S.E.M of *A. tumefaciens* attached on PLBs surface. Network of fibrils glued the bacteria to one another and anchored them to the plant surface (arrow).

the plant tissues, is mediated by the bacterial flagellar motility. The tight bond is also attributed to cellulose-containing microfibrils surrounding the bacteria, synthesised solely by the virulent bacteria and anchoring them firmly to the plant surface (Matthysse et al., 1981; Sreeramanan et al., 2009). The fibrils trap other surrounding bacteria to create a dense bacterial accumulation (Matthysse et al., 1981). Matthysse et al. (1981) also observed that plant cells were not

integral in bacterial attachment when the bacteria produce fibrils, as the bacteria were able to attach in the absence of live carrot cells. *Agrobacterium* attachments were initially observed on site-specific wounded plant cell surfaces (Matthysse, 1981). The establishment of injuries on plant cells aids in the release of acetosyringone for stimulation of bacterial virulent genes necessary for the T-DNA transfer, and in the provision of many bacterial entry points into the plant cell (Finer, 2010). This study indicated that PLB-wounding was not necessary for bacterial attachment, but aided in its enhancement. Escudero and Hohn (1997) showed that wounding-provoked responses were not essential for bacterial attachment but enhanced the process when conducted for the transfer or integration of the T-DNA in tobacco.

Materials and methods

Selection of PLB size and wounding condition

Protocorm-like bodies of *Dendrobium* sonia-28 were maintained in semi-solid half-strength MS (Murashige and Skoog, 1962) medium, containing 2% (w/v) sucrose and 1mg.L^{-1} 6-benzylaminopurine (BAP) at pH 5.75±0.05. The PLBs were incubated at 25±2°C under 16 hours photoperiod for four weeks and subcultured at least twice prior to use in the following treatments. Two groups of PLB sizes commonly found in the fourth week of culture were selected: large (3-4 mm) and small (1-2 mm). The PLBs were either wounded by transverse cutting or unwounded (control treatment). The wounding conditions were applied on both PLB size groups.

Bacteria preparation

Bacteria preparation was carried out using a protocol forwarded by Sreeramanan et al. (2009) with modifications. *Agrobacterium tumefaciens* strain LBA4404 harbouring the pCAMBIA 1304 plasmid and *Escherichia coli* strain DH5α harbouring the pMRC 1301 plasmid were cultured in Luria Bertani (LB) broth containing 50mg.L^{-1} kanamycin. The *A. tumefaciens* carried the β-glucuronidase (*gusA*) and kanamycin (*kan*) resistance genes, while the *E.coli* carried the *nptII* and *gusA* genes. The bacteria were grown overnight (28±2°C) at 120 rpm under dark condition. The bacterial culture was streaked on LB agar containing 50mg.L^{-1} kanamycin and incubated for 48 hours, at 28±2°C under dark conditions. Single colonies of bacteria were suspended into 30mL LB broth supplemented with 50mg.L^{-1} kanamycin. The bacteria were grown overnight at 28±2°C and 120rpm in the dark. The OD_{600nm} of the bacteria suspension was adjusted to 0.7 prior to use in the treatments. The *E. coli* culture was used as the control in the *Agrobacterium* attachment study.

Chemotaxis assay

The chemotaxis test was conducted using Shaw's swarming plate protocol (1995). The chemotactic medium contained 10mM potassium phosphate buffer (pH 7.0), 1mM ammonium sulfate, 1mM potassium-EDTA and 0.2% (w/v) bacteriology agar, topped up with LB broth. The bacteria were inoculated in the middle of the Petri plates using sterile 0.1mL pipette tips. Each PLB was cultured at a distance of 2.5cm from the inoculation point (Plate 3.3a). The PLBs were incubated at 28±2°C in the dark for 24 and 48 hours. The distance of bacterial movement, both towards and backward from the point of inoculation, was measured after 24 hours and 48 hours. The ratio of bacterial chemotaxis towards the PLBs was quantified using the following formula:

$$Chemotaxis\ Ratio\ (Unit) = Towards / Backwards$$

Where, towards = the distance of bacterial movement towards the explant exudate, backwards = the distance of bacterial movement away or backward from the explant exudate.

Quantification of Agrobacterium attachment

Bacterial attachment quantification was carried out following the protocol by Perez-Hernandes (2000) with modifications. Selected PLBs were maintained in 1mL 25mM phosphate buffer (pH 7.5). The PLBs were then transferred into 1.5mL Eppenddorf centrifuge tube containing 1mL fresh buffer and infected with 50µL of buffer-suspended bacteria. The explants were incubated for two hours at 28±2°C at an orbital speed of 40rpm. The PLBS were suspended, washed and vortexed for 10 seconds in 1mL fresh buffer, with the procedure performed twice. The buffer was completely removed, and 1mL of extraction buffer [50mM sodium phosphate buffer (pH 7.0); 10mM dithiothreitol; 1mM sodium EDTA; 0.1% (v/v) sodium lauryl sarcosine; and 0.1% (v/v) Triton X-100] was added into the tube. After vortexing for 30 seconds, the extraction was incubated at 37°C for 10 minutes. P-nitrophenyl β-glucoronide was added at a final concentration of 1mM into the tube, which was then vortexed for 10 seconds and incubated in 37°C for 30 minutes. Finally, the reaction was terminated by adding 400µL of 400mM disodium carbonate solution into the mixture. The absorbance of GUS expression was measured at A_{415nm}. A set of uninfected PLBs were prepared in the same way, eliminating the bacterial infection step, to measure the total explant exudate released in the reaction. Another set of PLBs infected with *Escherichia coli* was also prepared in the same way to be used as the negative control. The bacterial attachment percentage was quantified as the following:

$$Percentage\ of\ attachment\ (\%) = (A - B) \times 100/C$$

Where,
A = absorbance value of infected tissue at 415nm
B = absorbance value of uninfected tissue at 415nm
C = total bacterial inoculum
For microscopy observations of bacterial attachment on the PLBs, the PLBs were prepared according to a protocol by Hernandez (2000). The PLBs were freeze-dried with liquid nitrogen and coated with gold before observations under a scanning electron microscope (SEM).

Statistical analyses

Statistical analyses were performed using SPSS 16.0. The means were compared using one-way ANOVA and the differences contrasted using Tukey's multiple range test, at 5% significance level.

Conclusion

The chemotaxis and attachment studies revealed that 3-4mm PLBs of *Dendrobium* sonia-28 are favourable targets for transformation. Wounding, unnecessary in attracting *Agrobacterium*, is integral in the attachment of the bacteria on the PLBs. In conclusion, *Agrobacterium tumefaciens* exhibit positive bacterial-plant interaction towards

Dendrobium sonia-28, which could possibly encourage the production of transgenic orchids through the transformation method.

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