ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 5 (2): 198-202, 2006
ISSN 1682-296X
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Chemotaxis Movement and Attachment of *Agrobacterium tumefaciens* to Banana Tissues

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Abstract: An early step in the *Agrobacterium*-mediated transformation of banana was investigated in the plant-bacterium interaction. Chemotaxis of *Agrobacterium tumefaciens* strains (EHA 101 and LBA 4404) towards wounded banana tissues has been studied 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 banana under natural conditions. The spectrometric GUS and GFP assays provide information on the amount of inoculated *Agrobacterium tumefaciens* that effectively bound to banana explants. It can be concluded that *Agrobacterium* is able to attach specifically to different types of banana cells, which establishes the basic for a possible *Agrobacterium*-mediated transformation of banana cells.

Key words: *Agrobacterium tumefaciens*, chemotaxis, bacterial attachment, tissue culture

INTRODUCTION

Bacteria chemotaxis is considered as the first step in the interaction between *Agrobacterium* and plant cells during the process of bacterial infection (Perez Hernandez *et al*., 1999). Attraction of *Agrobacterium* towards plant exudates may account for its prevalence in the rhizosphere whereas the highly sensitive response to wound exudates may help guiding it directly to wounded plant cells (Shaw, 1991). Plant exudated compounds negatively affecting chemotaxis could be then at least partially responsible for the restricted host range of *Agrobacterium* in nature. Banana plants are unable to form crown gall tumors after *Agrobacterium* infection *in planta* or *in vitro* excised tissues (Perez Hernandez *et al*., 1999). Therefore, chemotaxis of *Agrobacterium* towards banana wound exudates has been studied to investigate in 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 repellant, respectively (Mao *et al.* 2003). In *Agrobacterium*, chemotaxis plays an important role during the early events of plant–microbe interaction (Perez Hernandez *et al*., 1999), since without it the cell-cell contact, which is required for DNA transfer, could not be established. Provided by peritrichous motility, *Agrobacterium* responds to the presence of plant exudates to direct itself to the vicinity of plant wounds. A chemotaxis cluster has been described in a region of the *Agrobacterium* chromosome which resembles chemotaxis operons identified in other members of the same group of proteobacteria (Wright *et al*., 1998).

Method for measuring chemotaxis uses swarm agar plates to quantify spatial movement of bacteria (Perez Hernandez *et al*., 1999). When inoculated onto the center 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 and built up by diffusing 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 behavior 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 *Agrobacterium tumefaciens* to target plant cells is essential for tumorigenesis and

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appears 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).

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). Therefore, it would be of interest to know whether the same pattern can be found during *Agrobacterium*-banana interaction, before attempting transformation in this species. Though attachment of *Agrobacterium* to plant cells can be observed through a number of microscopy techniques, the specificity of the cell-cell contact would be preferably demonstrated by a quantitative measurement of the binding capacities of attachment competent bacteria. The present study describes the study of *Agrobacterium tumefaciens* attachment to banana cultivar, Rastali (AAB) cells and tissues, using the quantification of bacterial attachment through the spectrophotometric GUS and GFP assays.

MATERIALS AND METHODS

Plant materials: Leaf, corm slice and root tissues were excised from *in vitro* plants of the banana cultivar, Rastali (AAB). Single buds (3 mm) were excised from multiple buds as described Sreeramanan *et al*. (2002). Callus were obtained from corm slice using 2 mg L^{-1} of picloram and harvested after 2 months. The leaf, corm slice, single buds and root tissues were used for chemotaxis assays. For quantification of bacteria attachment experiments, leaf, corm slice, single buds, callus and root tissues were used. Single buds were used for transient expression studies in transformation.

Bacterial strains: *Agrobacterium tumefaciens* strains, super-virulent strain EHA 101 (pIG 121-Hm) LBA 4404 (pCambia 1304) and *Escherichia coli* strain DH α (pMRC 1301) were maintained at -70°C for long term storage in 70% (v/v) glycerol. *Agrobacterium tumefacien* strain EHA 101 contained the disarmed plasmid pIG 121-Hm with the eukaryotic *npt 11* gene, which confers resistance to kanamycin and *hpt* genes for resistance to the antibiotic hygromycin, as well as an intron containing β-glucuronidase (*gusA*) gene. The LBA 4404 strain contained the disarmed plasmid pCambia 1304 differed from pIG 121-Hm by having the green fluorescence protein (*gfp*) gene. *Escherichia coli* strain DH α contained plasmid pMRC 1301 with *npt 11* and β-glucuronidase (*gusA*) genes only. However, this strain is used only as control bacteria for the quantification of *Agrobacterium* attachment studies. Cultures were grown from single colonies in Luria-Bertani (LB) medium by incubation at 28°C and 120 rpm for 20 h to reach an optimal density of 0.7 units at 600 nm (OD_{600}). Appropriate antibiotic was included in the medium at the following concentration: kanamycin (50 mg L^{-1}).

Chemotaxis assays: Chemotaxis assays were carried out according to the modified swarm agar plate method of (Perez Hernandez *et al*., 1999). 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. Excised but otherwise intact explants (first level of wounding: W_1), explants chopped into small pieces (second level of wounding: W_2) and explants that were bombarded (third level of wounding: W_3) were used for this assay. The tissues were bombarded at (1100 psi) with naked gold particles at a distance of 9 cm under vacuum pressure (~28 mm Hg). The preparation and experimental details for particle bombardment are as described by Sreeramanan *et al.* (2005). Chemotaxis was quantified after 24 h incubation at 28°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 four replicates each.

Quantification of bacterial attachment: Quantification of bacterial attachment assays were carried out according to method of Perez Hernandez *et al*., 1999). Banana leaf, corm slice, root, single bud explants and callus were prepared as follows: leaf discs and 2 mm thick corm disks excised from sliced corms were prepared using a 5 mm diameter cork borer; single buds of approximately 3 mm in size were separated from a multiple bud clumps; roots were cut into 2 cm long segments and callus tissue weighing for 4 mg 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 LBA 4404, the *Escherichia coli* strain DH α was included in all experiments as negative

controls. For infection, 1.5 mL Eppendorf tubes filled with 1 ml of the same buffer were loaded with either four leaf disks, one corm disk, four root segments, four single buds or 4 mg callus, to which 50 μL aliquots of buffer suspended bacteria were added. Tubes were then incubated in a rotary shaker at 28°C at 25 rpm for 2 h. After this period, unbound bacteria were removed by washing the explants twice with 1 mL fresh buffer and vortexed 30 sec each time to discard unattached bacteria. β-Glucuronidase activity in the samples was measured following 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 min. The GUS enzyme substrate p-nitrophenyl α-D-glucuronide was added at a final concentration of 1 mM, and after incubation at 37°C for 30 min, reactions were stopped by the addition of 400 μL of a 400 mM Na_2CO_3 solution. GUS activity was quantified by measuring light absorbance at 415 nm (A_{415}). Absorbance was also measured from explants containing uninfected tubes to determine levels of light absorption at 415 nm from plant released compounds, as well as from inoculated tubes in the absence of plant material to record the total enzymatic activity in the inoculum used for interactions. For 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 light absorbance at 510 nm (A_{510}) 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.

Statistical analysis: Data were analysed using one-way ANOVA and the differences contrasted using Duncan's Multiple Range test. All statistical analyses were performed at the level 5% using SPSS 10.0 (SPSS Inc. USA).

RESULTS AND DISCUSSION

Chemotaxis of *Agrobacterium tumefaciens:* 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 LBA 4404, results in the aggregation of bacterial cells in culture and makes it difficult to accurately quantitate 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 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 banana explants and bacterial strains tested, independently of the wounding level. The overall swarming ratio of the two bacterial strains tested in the presence of banana tissues ranged between 1.82 and 1.25 indicating a positive effect of the plant exudates on bacterial movement. This effect is shown for the two strains EHA 101 and LBA 4404 after assaying with excised tissues tested (Fig. 1). In many cases, bacterial migration to banana 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 LBA 4404 in the presence of single buds and corm slices were always higher with the increased level of wounding (Fig. 1).

However, there is 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 1.5, LBA 4404 usually migrated faster towards the explants, which resulted in chemotaxis ratios over 1.70 units. Results obtained from the root and leaf tissues showed a weak positive chemotaxis response suggest that these explants is could be least partially responsible for the restricted host range of *Agrobacterium* in nature.

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

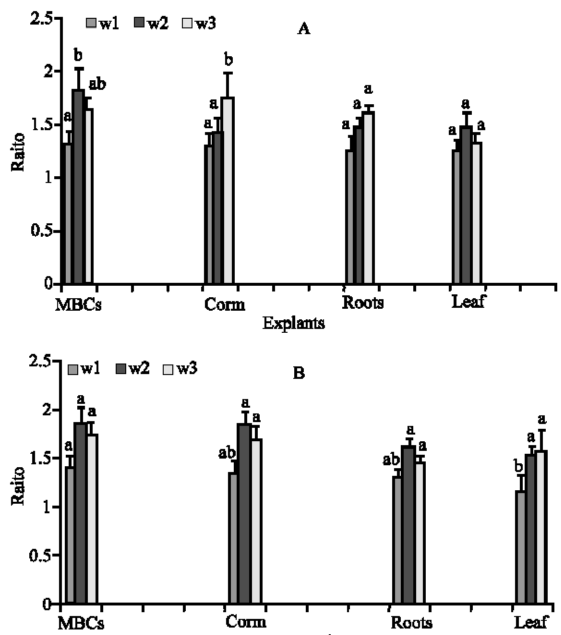
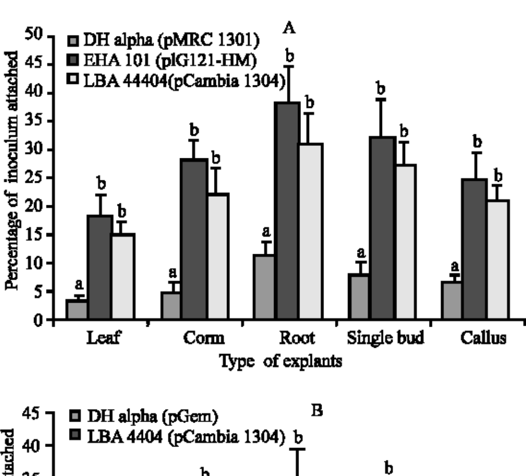


Fig. 1: Chemotaxis ratios of *Agrobacterium tumefaciens* strains EHA 101 (Panel A) and LBA 4404 (Panel B) in the presence of a single buds (3 mm), corm slice, root and leaf at low (W_1) or increased (W_2 and W_3) wounding level. All experiments were repeated for four times. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

maize exudates. Positive chemotaxis toward root and shoot homogenates from the dicotyledonous host *Kalanchoe* sp. and the non-host monocotyledonous wheat plants suggested that both plants have equally strong chemoattractants for *Agrobacterium tumefaciens* (Ashby *et al*., 1988).

Quantification of bacterial attachment: Besides the chemotaxis, 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. 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 LBA 4404 over the DH α of the naturally non-attaching *E. coli* (Fig. 2).

Whereas no major differences were observed among the two *Agrobacterium* strains tested, attachment to root



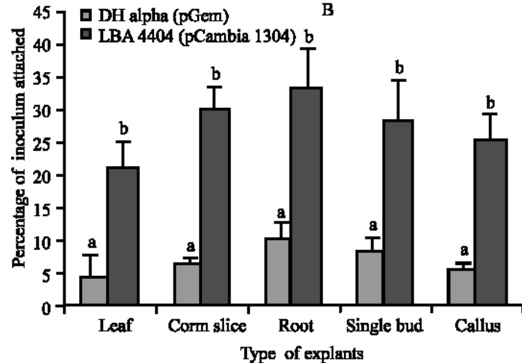


Fig. 2: Quantification of bacterial attachment to banana leaf, corm slice, root, bud and callus tissues through spectrophotometric measurement of GUS (Panel A) and GFP (Panel B) expressions in genetically marked bacteria. Values correspond to the percentages of inoculated bacteria remain attached to cells after extensive washing of infected tissues. Data were analysed using one-way ANOVA and the differences contrasted using Duncan's multiple range test. Different letters indicate values are significantly different ($p<0.05$)

explants showed the greatest differences compared to binding-deficient bacteria, *E. coli* (control bacteria) using GUS expressions (Fig. 2; Panel A). Due to the presence of the root hairs, root 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 leaf, corm slice, single bud and callus, 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. Both GUS and GFP expressions revealed highest percentage of inoculum attached in root explants (Fig. 2).

Therefore, root explants provide a better system for studying bacterial attachment to plant cells. Using this type of tissue, bacterial attachment to wheat, maize and pea 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). Similarly to what was observed in the case of banana cultivar, Rastali (AAB), the superior binding ability of the attachment–competent *Agrobacterium* strains was also evidenced.

CONCLUSIONS

It can be concluded that *Agrobacterium* is attracted to exudates from banana 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. The quantification system proved that *Agrobacterium* is able to attach specifically to different types of banana cells based on the GUS and GFP spectrometric assays.

ACKNOWLEDGEMENTS

The authors wish to thank Tsukazaki Hikaru from National Institute of Agrobiological Resourses, Japan, for the EHA 101 (pIG 121-Hm) and Dr. Richard I.S. Brettell from CSIRO, Australia for the LBA 4404 (pCambia 1304). This research was supported financially by The Ministry of Science, Technology and Environmental (MOSTE) through the PR project (01-02-04-0060-PR0010/041).

REFERENCES

Ashby, A.M., M.D. Watson, G.J. Loake and C.H. Shaw, 1988. Ti-plasmid specified chemotaxis of *Agrobacterium tumefaciens* C58 toward *vir*-inducing phenolic compounds and soluble factors from monocotyledonous and dicotyledonous plants. J. Bacteriol., 170: 4181-4187.

Hawes, M.C., L.Y. Smith and A.J. Howarth, 1988. *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. Molecular Plant-Microbe Interactions, 1: 182-186.

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

Mao, H., P.S. Cremer and D. Manson, 2003. A sensitive, versatile microfluidic assay for bacterial chemotaxis. Proc. Natl. Acad. Sci. USA., 100: 5449-5456.

Perez Hernandez, J.B., S. Remy, Galan V. 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.

Shaw, C.H., 1991. Swimming against the side-chemotaxis in *Agrobacterium*. BioEssays, 13: 25-29.

Shaw, C.H., 1995. *Agrobacterium tumefaciens* Chemotaxis Protocols. In: Gartland, K.M.A. and M.R. Davey, (Eds.). Methods in Molecular Biology. Vol. 44. *Agrobacterium* Protocols, pp: 29-36.

Sreeramanan, S., M. Marziah and Mohd Puad Abdullah, 2002. Optimization of multiple buds production in Pisang Rastali (*Musa sapientum* cv. Rastali). In: Proceedings of the 3rd National Banana Seminar, Port Dickson, Negeri Sembilan, Malaysia, pp: 31.

Sreeramanan, S., M. Maziah, M.P. Abdullah, M. Sariah and M. Nor'Aini, 2005. Physical and biological parameters affecting transient GUS and GFP expressions in banana via particle bombardment. Asia Pac. J. Mol. Bio Biotechnol., 13: 35-57.

Vergauwe, A., Van W. 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.

Wilson, K.J., S.G. Hughes and R.A. Jefferson, 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: Gallagher, S.R. (Ed.). GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression, pp: 7-22.

Wright, E.L., W.J. Deakin and C.H. Shaw, 1998. A chemotaxis cluster from *Agrobacterium tumefaciens*. Gene, 220: 83-89.