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ATTACHMENT STUDY OF *AGROBACTERIUM TUMEFACIENS* TO TEF [*ERAGROSTIS TEF* (ZUCC.) TROTTER], YAM SPP., AND TOBACCO (*NICOTIANA TABACCUM* L.) EXPLANT

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ABSTRACT: *Agrobacterium tumefaciens* attachment as a factor in transformation was investigated in tef (*Eragrostis tef*) zygotic embryos, seeds, seedlings, leaf bases and embryogenic callus; leaf discs of yam species (*Dioscorea bulbifera, Dioscorea caynensis* and *Dioscorea alata*); and leaf discs of tobacco (*Nicotina tabaccum*). Cocultivation was made with A1030(pCOPA10), A1030(pEB122), A1030(pTBl08), A1030(plB422), A1030(virA:Tn5) and A281(pTiBO542). Attachment and fibrils were observed associated in all strains, species, plants and explant used. Even though attachment is observed in intact and wounded plants, preferential attachment was observed around the wound area of explants. Uniformity with bacteria binding was obtained with acetosyringone treatment. The strains used equally bound to tef and yam in the same way as they did to tobacco which was a positive control. Thus, tef and yam, like that of tobacco fit all the criteria for Agrobacterium attachment.

Key words/phrases: Acetosyringone, *Agrobacterium tumefaciens*, plant-bacterial attachment, tef, scanning electron microscopy

INTRODUCTION

Agrobacterium is a routine and efficient method for the production of transgenic plants from numerous non-cereal species (Potrykus, 1980). Non-cereal species without wound response are not easy to transform. Transformation of monocots with a wound response, for example, asparagus and yam are as easy to transform as dicot with wound response. In most monocotyledon plants, the wound response leads to lignification and sclerification of the wound adjacent cells in the absence of apparent cell divisions. Wound healing responses in monocotyledons is observed both through mechanical injury and chemical treatments (Swamy and Sivaramakrishna, 1975).

The efficiency of T-DNA transfer by *Agrobacterium* in monocots and dicot is equal as has been shown by *Agrobacterium*-mediated delivery of infectious maize streak virus (Grimsley *et al.*, 1987).

One of the earliest stages of interaction between *Agrobacterium* and plants is the attachment of the bacteria through plant surfaces. Such binding can be observed by either light microscopy, or scanning electron microscopy (Matthyse *et al.*, 1981).

Cells of several monocot species bind fewer virulent *Agrobacterium* than do dicot plant cells, but also monocots notably corn and brome grass (*Bromus inermis*) bind as many bacterial cells as does *D. innoxia* (Lippincott and Lippincott, 1975).

Monocot cell walls were thought to have fewer bacterial attachment sites than dicots, which would account for the lack of tumour formation in some monocots. It has been reported that more of these bacteria attach to carrot than to corn or oat protoplasts from suspension cultures (Matthyse *et al.*, 1981) and they do not attach to embryonic leaf fragments of maize (Lippincott and Lippincott, 1975).

Site specific attachment of *A. tumefaciens* to host cells is an essential stage in crown gall tumour genesis. *Agrobacterium* mutants that are defective in attachment are either avirulent or severely attenuated in virulence and this is also consistent with the hypothesis of site specific attachment (Congelosi *et al.*, 1981). Accordingly, attachment site specificity, or otherwise, is important for virulence. However it is indicated that there is no evidence that virulent *Agrobacterium* cells preferentially bind to damaged cell walls (Lippincott and Lippincott, 1975). *A. tumefaciens* attachment presumably involves the interaction of one or more of its surface molecules with plant cell wall.

To date, three genetic loci have been defined as having roles in *A. tumefaciens* attachment to plant cells: *chv*A and *chv*B (Douglas *et al.*, 1982) and *pSCA* (exoC) (Congelosi *et al.*, 1981). Random screen for *Tn*5 induced mutations resulted in the isolation of *chv*A and chvB mutants. Direct binding assays indicated that these mutants were markedly reduced in their abilities to attach to both tobacco tissue culture cells and freshly isolated mesophile cells of zinnia (Douglas *et al.*, 1982).

Lippincott and Lippincott (1975) and Lippincott *et al.* (1980) reported that complementary plant and bacterial receptors are involved in site attachment. Therefore the objectives of this study were: to assess whether attachment is a factor for *Agrobacterium* based gene transfer system; to evaluate attachment interaction of bacterial strains and crop types; and to compare and contrast monocots, tef and yam with that of tobacco for attachment.

MATERIALS AND METHODS

Agrobacterium *strains, plants, species and explant*

Six *Agrobacterium* strains were used for the attachment study and their characteristics is indicated (Table 1). Plant species and explants consisted of tef (*Eragrostis tef*) callus, seedling, leaf base, seed, zygotic embryo; tobacco (*Nicotiana tabaccum*) and yam species leaf discs.

Table 1. Agrobacterium strain characteristics.

Strains	Characteristics
A1030 (*vir*A:*Tn*5)	*vir*A inactivated, Km^r
A1030(pEB122)	*vir*A^{con} mutants, Km^r, cb^r
A1030(pCOPA10)	*vir*A^{con} mutants, Km^r and cb^r
A1030(pTB108)	wild type, *vir*A gene, Km^r,cb^r
A1030(pIB422)	wild type, *vir*A gene, Km^r,cb^r
A281 (pTiBO542)	hyper virulent strain, WHR agropine type

Con, constitutive; Km, kanamycin; Cb, carbenicillin; WHR, wide host range; r, resistance.

Infection treatment

Single colonies of bacterial strains A1030 (virA:Tn5), A1030 (pEB122), A1030(pCOPA10), A1030 (pTB108),A1030 (pIB422) and A281(pTiBO542) were inoculated into 10 ml luria-broth (Sigma) containing 100 µg/ml of Kanamycin and Carbenioillin as per the requirement of the strain, and grown for 12 hr. on an orbital shaker from 27 to 30° C. Bacterial inoculation was transferred to 15 ml centrifuge tubes and centrifuged for 10 min. The cells were washed three times with 0.85% (w/v) NaCl. After an optical density reading at 620 nm wavelength, it was diluted to a titer of 10^8 cells/ml using salts liquid medium of Murashige and Skoog (1962). Explants were sterilized with an initial 3 minutes treatment in 70% (v/v) enthanlo followed by a 30 min. wash in 40% (v/v) in commercial bleach containing 0.1% (v/v) tween and sterile embryogenic callus was made available from tissue culture study of tef (Frew Mekbib *et al.*, 1997). Tef, tobacco, and yam explants were used. The latter two served as dicot and monocot positive controls, respectively.

The explants were treated differently as unwounded, wounded, and wounded plus 500 um of acetosyringone in dimethylsulfoxide (DMSO) and co-cultivated with strains of agrobacteria and grown overnight in eppendorf tubes for 12 hrs on a shaker at a rate of 150 rpm for uniform attachment at 27°C. They were then washed vigorously twice by vortexing for 30s in phosphate-buffered 0.9% saline solution (9 g of NaCl, 2.7 g of Na_2HPO_4, 0.43 g

of H_2PO_4 in 1 L of distilled water) at pH 7.2 to remove any bacteria that were not firmly attached (Graves *et al.*, 1988).

Scanning electron microscopy

For studying degree and orientation of attachment, different infected explants were prepared for electron microscopy using standard fixation technique for biological materials. The procedure used was as follows: tissues were fixed in 0.05 M phosphate buffer containing 2.5 % gluter aldehyde in acetone solutions of 50, 70, 80, 90% (v/v) for 30 min. The final dehydration was made three times in 100 % acetone (stored over desiccant) for 15 min twice and left overnight. Specimens were critically point-dried after mounting them in araldite or colloidal silver on metal "stubs" and then sputter coated for 4 min with a very thin layer of gold and viewed with Hitachi S430 Scanning Electron Microscope.

RESULTS AND DISCUSSION

The initial attachment of single bacterial cells is often in polar fashion followed by massive aggregation of single bacterial cells at the plant cell surface (Fig. 1a,b,c). The first step in tumor formation by *Agrobacterium tumefaciens* is, *in vivo,* site specific binding of the bacteria to plant host cells (Lippincott and Lippincott, 1969). Attachment is followed by transfer of *Ti* plasmid and the initial attachment is often in polar fashion.

Following attachment to the cell, the bacterial cells synthesized fibrils which served to anchor the bacteria to the surface of the plant cells and also caused entrappment of other bacterial cells.

Though, after attachment, the bacterial cells synthesized fibrils, cellulose-deficient mutants did attach to plant cells which indicated that fibrils were not required for attachment *per se*. Even though it has been suggested that bacteria produce large quantities of cellulose fibrils which cause clamping of the bacterial cells at these sites after they are induced by the presence of wounded cells, fibrils were observed both in mechanically wounded and intact plant cells. These cellulose fibrils apparently serve to anchor the bacteria to the host cell surface after initial binding has occurred (Matthyse *et al.*, 1981).

Delicate fibrils were observed associated with the bacteria attached to both mechanically wounded and intact plant cells. The bacteria are attached by fibrillar connections not only to intact or wounded surfaces, but also to each other as well (Fig. 2). Thus it would appear that binding is not wound dependent.

Fig. 1a. Attachment in Polar orientation of A1030 (pCOPA10) to intact tef zygotic embryos. Note the fibrillar attachment which are arrowed (x 52,000).

Distribution of attachment varies with wounded and unwounded sites, and also in different explant. The bacterial cells were found to be thinly distributed over the surface of the unwounded explant. High levels of uniformity of attachment were observed in wounded plus acetosyringone (AS) treatment (Fig. 3). AS is a primary signal for agrobacterium-plant attachment and it is commonly synthesized and exudated by metabolically active wound cells. It is a natural component of plant cells and thus affects the binding property of plant cells. In contrast, in the wounding treatment, the bacterial cells were found to be aggregated at the site of maximal wounding (Fig. 4).

Presupposing that the fibrils observed in the present study were the same type as those observed by (Matthyse *et al.*, 1981), it follows that the cellulose fibril synthesized by *Agrobacterium* is not wound dependent, but is more likely to be dependent on plant/bacterial site recognition. Since fibril synthesis was not dependent upon the attachment at a wound site or the presence of wounding, synthesis of fibrils is also not dependent upon the presence of wounding factor.

Fig. 1b. Attachment in Polar orientation of A1030 (Vir A:Tn 5) to *D. bulbifera* (x 20,100).

Fig. 1c. Attachment in Polar orientation of A281 to tef leaf base (x 25,000).

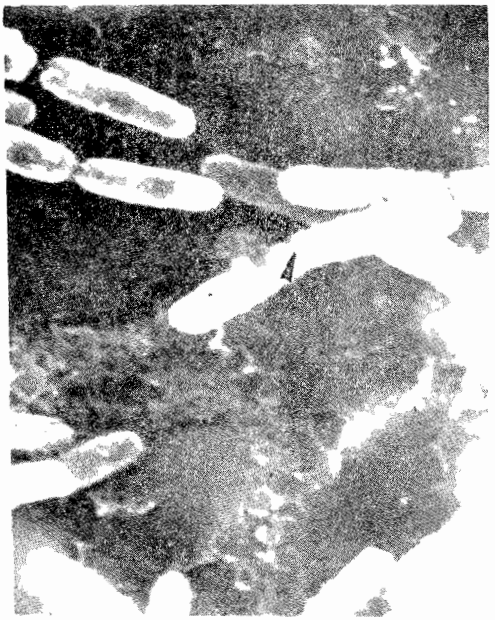

Fig. 2. **Bacteria attached to each other by fibrils A1030(pCOPA10) attached to wounded *Dioscorea caynensis*. Fibrils arrowed. (x 6,200).**

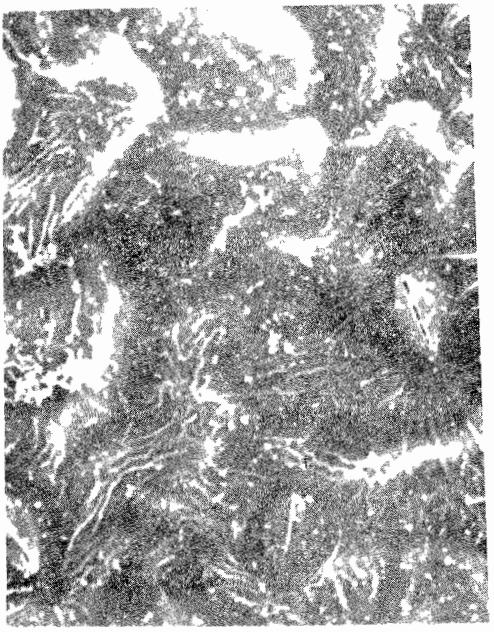

Fig. 3. **Uniformity of attachment of A1030 (pCOPA10) on tef seed embryonic surface with the addition of 500 um of acetosyringone. (x 1600).**

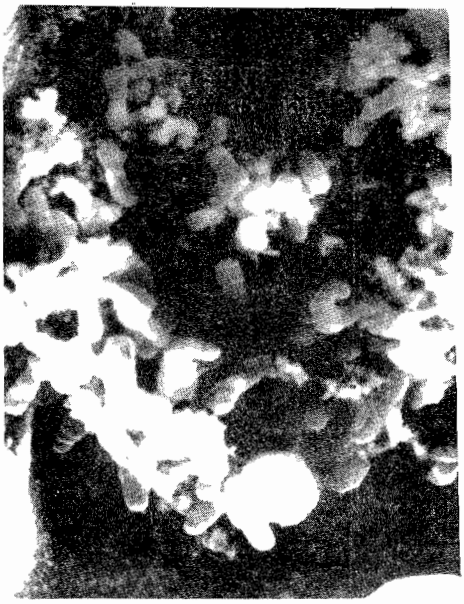

Fig. 4. Preferential attachment and aggregation of A281 on wounded leaf discs of *Dioscorea alata* cv. Lisbon (x 5,000).

There seems to be equal degree of attached bacterial cells to both mechanically wounded and intact explant. There was increased attachment with acetosyringone treatment. Preferential attachment to the sites of wounding was consistent in all species considered and explant used. Aggregation of bacterial cells at the site of maximal wounding surface suggests that the wound site or a chemical factor associated with site, focuses the bacteria towards it, thereby creating niche to which the bacteria preferentially attach.

Increased binding of *Agrobacterium* was observed in tef seedlings (coleoptile, mesocotyle, crown), leaf discs and zygotic embryo rather than in seeds. Higher levels of binding in the seed were observed in the embryonic surface. Attachment has been found to tef proglobular embryonic calli surface (Fig. 5).

An apparent lack of a low rate of attachment to monocotyledonous crop has in the past been attributed in part to partial methylation of the binding sites on the cell walls. If methylation of the binding sites on the cell wall has occurred in the present study, the problem has not been marked as previously reported. The bacteria did not induce tumor on monocotyledons or meristematic dicotyledon tissues (Lippincott and Lippincott, 1969). Therefore, one factor in determining host range of *A. tumefaciens* may be the presence or absence of a number of accessible bacterial binding sites on the plant surface. In case of tef,

a high level of attachment has been obtained in meristematic, embryonic surface of seedlings and explants used.

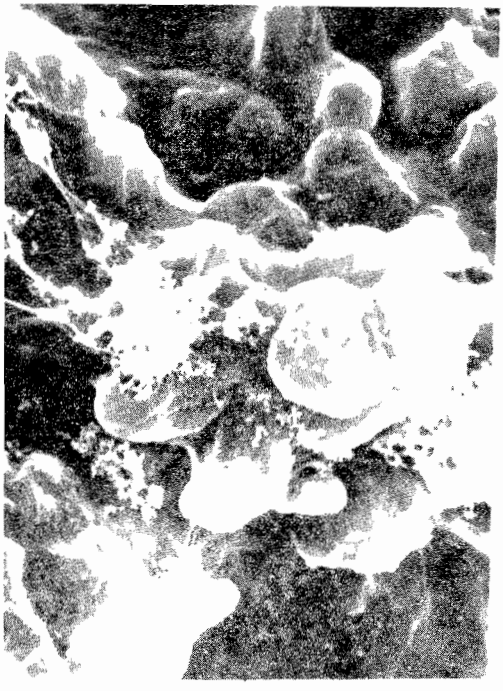

Fig. 5. A281 attached to proglobular embryogenic callus of tef (x 1,000).

Other workers reported that enhanced attachment of *Agrobacterium* cells to the surface of carrot cells following partial enzymatic digestion of the cell wall (Matthyse and Gurlitz, 1982). However, the total number of bacteria that attached to partially digested carrot cells was higher than those attached to intact carrot cells. Our findings, however, showed that wounded and intact explant showed similar attachment.

The orientation and degree of attachment of *Agrobacterium* to *Dioscorea spp.* and tef was similar. The difference of *Agrobacterium* strains in attachment to the two plant species was very minimal. The strains A1030 (virA:Tn5) (Fig. 6 and 1b), A1030 (pCOPA10) (Fig. 1a), and A281 (Fig. 4 & 1c) showed no apparent difference in their competence to attachment .

Therefore, attachment of the bacteria to an intact cell wall may be a prerequisite for transformation. Thus, the monocots, tef and yam spp., like

that of model crops, fit all the criteria for *Agrobacterium* attachment and hence the first system in the *Agrobacterium* gene transfer.

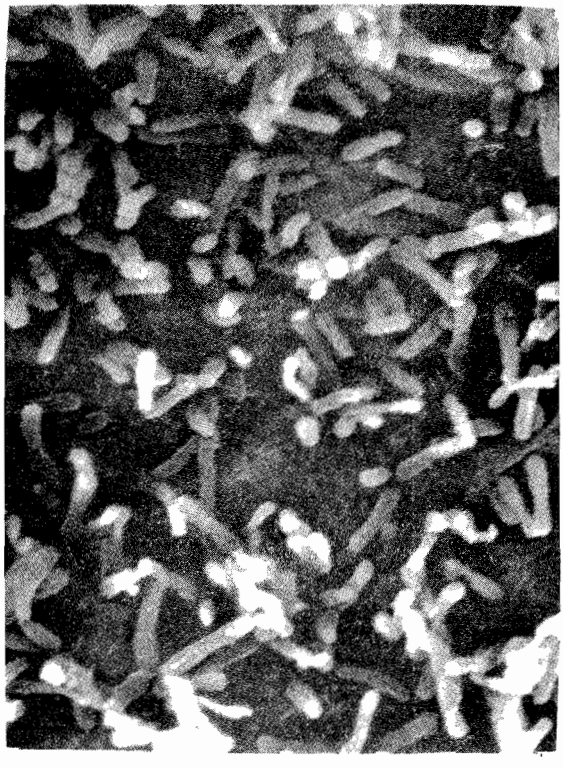

Fig. 6. A1030 (*vir* A:Tn 5) attached to tobacco leaf discs (x 7,000).

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