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Evidence for the involvement of exposed surface carbohydrates of *Agrobacterium tumefaciens* in relation to crown gall tumour induction

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Abstract. The exposed carbohydrate residues on the cell surface of both tumourigenic and nontumourigenic strains of *Agrobacterium* have been investigated using lectins as probes. N-acetyl-D-galactosamine and β-D-galactose were found to be present as the exposed groups on the cell surfaces of he *Agrobacterium* strains. These carbohydrate residues are attached to lipids on the outer membrane of the bacteria as lipopolysaccharides. Fluorescently labelled lectins were used to observe bacterial agglutination in fluorescence microscope. The involvement of these exposed carbohydrate groups in host-pathogen interaction was demonstrated by actual inhibition of tumour initiation at wound sites on host plant.

Keywords. β-D-Galactose; N-acetyl-D-galactosamine polygalacturonate; adherence; lectin binding; tumour inhibition (*Agrobacterium tumefaciens*).

Introduction

Agrobacterium tumefaciens induces crown gall tumour in a wide variety of dicotyledonous plants. In order to be virulent *Agrobacterium* strains must harbour a tumour inducing plasmid (T_i) (van Larebeke *et al.,* 1974; Watson *et al.,* 1975; Thomashow *et al.,* 1980). This plasmid is introduced into the host plant genome for the induction and maintenance of tumours by the virulent strains (Chilton *et al.,*1977; Thomashow *et al.,*1980; Willmitzer *et al.,*1980; Yadav *et al.,* 1980). Lippincott and Lippincott (1977,1980) have shown that the primary event in crown gall tumour induction is the attachment of the bacterial cells to the host plant cell wall. This bacterial adherence has been confirmed by various groups of workers using different methods including electron microscopy (Manigault, 1970; Nissen, 1971; Matthysse,1978; Smith and Hindley, 1978 Ohyama *et al.,* 1979). Whatley *et al.,* (1976) have demonstrated that *Agrobacterium* component involved in the microbial-plant adherence is the polysaccharide or *O*-antigen portion of *Agrobacterium* lipopolysaccharide (LPS) by its ability to inhibit tumour induction. The lipid-A portion solubilized with bovine serum albumin is noninhibitory. This moiety anchors the LPS molecule in the outer membrane, while only the projecting polysaccharide chains participate in the adherence. On the part of the host cell wall, polygalacturonic acid has been implicated in the adherence mechanism because of its excellent ability to inhibit tumour induction. A similar situation prevails in the case of the other genera of *Rhizobiaceas i.e.* of *Rhizobium* species which induce

atmospheric fixing nodules in leguminous plants. Here, the bacterial polysaccharides occurring in the capsule or LPS portion of the envelopes which are antigenically very similar to polysaccharides of the host cell surface, are involved in adherence. Multivalent lectins of the host legume are envisaged to bind to each of these common antigens thereby leading to adherence (Hamblin and Kent, 1973 Bohlool and Schmidt, 1974; Dazo and Brill, 1978,1979). However, in the case of *Agrobacterium* plant interaction, no evidence for the participation of host lectins has been found. A direct polysaccharide-polysaccharide interaction occurs in the adherence of the *Agrobacterium* cells to the host plant cell wall. The polysaccharide groups of *Agrobacterium* cells participating in interaction have been reported (Banerjee *et al.*,1981). In order to confirm the involvement of N-acetyl-D-galactosamine and β-D-galactose in adherence, lectin binding studies and tumour inhibition studies were carried out.

Materials and methods

Lectins

The following plant lectins were used:

Lens culinaris (D-mannose, D-glucose) was prepared by the method of Howard *et al.* (1971); *Ricinus communis* (β-D-galactose) was purchased from Sigma Chemical Co., St Louis, Missouri, USA; *Dolichos biflorus* (N-acetyl-D-galactosamine) was prepared by the method of Etzler (1972) and *Ulex europaeus* (D-fucose) was prepared by the method of Osawa and Matsumoto (1972). The carbohydrate specificities of the lectins are given earlier (Thomashow *et al.,* 1980; van Larebeke *et al.,* 1974).

Organisms: The strains of *Agrobacterium tumefaciens* used were (i) TIP Kerr 14 agrocin sensitive,· referred to as '*N*' hereafter, (ii) TIP Kerr 14 agrocin resistant, probably cured of plasmid, referred to as '*R*' hereafter and (iii) S 1005 agrocin resistant variety, referred to as '*S*' hereafter. The above strains were kindly provided by Prof. J. Schell, Director, Laboratorium voor Genetica, Rijks Universiteit, Ghent, Belgium.

Growth Media: The organisms were grown in Rich yeast tryptone medium having the following composition 0.8% Bactotryptone, 0.5% Bactoyeast extract and 0.5% NaCl, pH 7.4. The growth temperature was 28°C. Cells from mid log phase were centrifuged at 700 *g* for 20 min in a refrigerated centrifuge (model B 20-A, IEC, Massachusetts, USA), washed twice in 0.1 M phosphate buffered saline (PBS) and resuspended in the same for use in agglutination tests.

Agglutination tests: Serial two fold dilutions of lectins in saline were performed in microtiter plates. Bacterial suspension (25 µl) was added to each well. After thorough mixing the plates were incubated at 37°C for 35 min. Agglutinations were observed and scored under light microscope. Agglutination titres of lectins are given as the reciprocal of the highest dilution, causing agglutination visible under a light microscope.

Pronase treatment: Cell suspensions were treated with 200 µg of pronase (Sigma Chemical Co., St. Louis, Missouri, USA) perml for 1 hat 37° C. This was then centrifuged at 7000 *g* for 30 min, washed twice with PBS and resuspended for agglutination tests.

Periodate treatment: Cell suspensions were treated with sodium periodate (Sigma Chemical Co., St. Louis, Missouri, USA) at a final concentration of 0.01 M in saline for 1 h at 37° C. This was then centrifuged at 7000 *g* for 30 min, washed twice in PBS, and resuspended for agglutination tests.

Inhibition of agglutination: Glucose, β-D-galactose, glucosamine, galactosamine, N-aceryl-D-glucosamine, N-acetyl-D-galactosamine, L-fucose and Lactose were obtained from E. Merck, Darmstadt and Sigma Chemical Co., St. Louis, Missouri, USA. Stock solutions of carbohydrates (1, 2 and 3%) were prepared in saline. Equal volumes of carbohydrate and lectin solutions were mixed and incubated at 37°C for 25 min. One drop of this mixture, was added to one drop of cell suspension, mixed, incubated and then observed under a light microscope.

Fluorescent labelling of lectins: Fluorescent labelling was carried out by the modified method of Clausen (1974) using fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, Missouri, USA). Agglutination of *Agrobacterium tumefaciens* cells by fluorescein isothiocyanate (FITC) labelled lectins were observed in a fluorescence microscope (Carl Zeiss, Jena, fluorescence microscope, NU-2 using an Osram HBO-200 light source and transmitted dark field tungsten illumination (Zeiss no. OG1 was the barrier filter while the excitor filter was B 223 g).

Tumour inhibition studies: Young healthy plants (*Bryophyllum calycinum* Salisb) with 2 to 4 internodes and stem of 5 cm diameter at the base were selected. The chosen sites on the stem were cleaned with distilled water and ethanol. Wounds were made with sterile needle by puncturing the stem. For inhibition studies the wounded areas were treated with the respective carbohydrate solutions according to the following protocol (table 1).

Table 1. Protocol for studying tumour inhibition in plants.

Carbohydrate used individually	Concentrations used in each case	Period of treatment	No. of plants per group
N-Acetyl-D-galactosamine, β-D-Galactose Lactose, N-acetyl-D-glucosamine Galactosamine Glucosamine Glucose	1.0 mg/ml 2.5 mg/ml 5.0 mg/ml	3 h 6 h 24 h 36 h	3
NaCl control	0.9%	36 h	3

For treatment, the wounded areas were covered with sterile gauze and carbohydrate solutions were applied by means of capillary tubes for the scheduled period of time. After the carbohydrate treatment, bacteria were introduced into these wound areas at a concentration of 10^8 cells/ml by means of a looped needle. The wounds were then covered with sterile cotton and the plants kept for three days at 25° C under alternate light (14 h) and dark (10 h) conditions. After this phase the cotton wrappings were removed and the plants were kept at room temperature (30°C). For control experiments, instead of carbohydrate treatment, 0.9% (w/v) NaCl was applied at wound sites. The rest of the procedure for tumour induction was the same as for the carbohydrate treated plants. Pea sized tumours (in controls) appeared after 15 days.

Results

The *Agrobacterium tumefaciens* cells were agglutinated by only two of the lectins used *viz.* lectins of *Dolichos biflorus* and *Ricinus communis,* indicating that the exposed carbohydrate residues are N-acetyl-D-galactosamine and β-D-galactose. The agglutination patterns, titers and results of the inhibition experiments are summarized in table 2. The sensitivity of the agglutination reactions towards

Table 2. Agglutination of *Agrobacterium tumefaciens, N R* and *S* strains by the lectins.

Agrobacterium tumefaciens strains	Agglutination by lectins [a]			
	L. culinaris	*D. biflorus* [c]	*U. europaeus*	*R. communis* [d]
N : intact cells	0	++ (32) [b]	0	++ (64)
pronase treated	0	++	0	++
periodate treated	0	0	0	0
R : intact cells	0	++ (32)	0	++ (64)
pronase treated	0	++	0	++
periodate treated	0	0	0	0
S : intact cells	0	++ (32)	0	++ (64)
pronase treated	0	++	0	++
periodate treated	0	0	0	0

[a] Intensity of the agglutination was recorded as: no agglutination O; strong agglutination ++.

[b] Titer given in parentheses is defined as the reciprocal of the highest dilution of lectins causing agglutination visible in a light microscope.

[C] Agglutination was inhibited by β-D-galactose (1%), lactose (2%), N-acetyl-D-galactosamine (1%), galactosamine (2%).

[d] Agglutination was inhibited by β-D-galactose (1%), N-acetyl-D-galactosamine (1%), galactosamine (2%), glucosamine (5%).

sodium periodate, indicates that carbohydrate structures are the lectin binding determinants. Pronase treatment did not influence agglutination, showing thereby that the carbohydrate residues are not attached to proteins but to lipids as LPS on the cell surface. Fluorescence microscopy using FITC labelled lectins of *Dolichos biflorus* and *Ricinus communis* confirmed agglutination of *A. tumefaciens* by these lectins. The results of tumour inhibition studies as presented in table 3 indicate that N-acetyl-D-galactosamine and β-D-galactose do indeed participate in adherence by their ability to inhibit tumour induction.

Discussion

Agglutination of both tumourigenic and nontumourigenic strains of *A.tumefaciens* by lectins of *D. biflorus* and *R. communis* indicates that the carbohydrate residues exposed on the cell surfaces are the same in all these strains. These results experimentally verify the observations of Lippincott and Lippincott (1969) that both

Table 3. Ability of different carbohydrates to inhibit crown gall tumour induction.

Carbohydrate used	Concentration in mg/ml	Ability to inhibit tumour induction after			
		3 h	6 h	24 h	36 h
N-Acetyl-D-galactosamine	1.0[a]	+	+	+	+
β-D-Galactose	1.0[a]	+	+	+	+
N-Acetyl glucosamine	5.0[b]	−	−	−	−
Glucosamine	5.0[b]	−	−	−	−
Galactosamine	5.0[b]	−	−	−	−
Glucose	5.0[b]	−	−	−	−
Lactose	5.0[b]	−	−	−	−
Control NaCl	0.9%	−	−	−	−

[a] These represent the minimum concentrations required for tumour inhibition.

[b] These represent the highest concentrations used.

strains can bind to plant cell walls. The carbohydrate residues exposed on the *Agrobacterium* cell surface *viz.* N-acetyl-D-galactosamine and β-D-galactose probably comprise the *O*-antigenic region of the LPS molecules on the surface. The *O*-antigenic region of LPS of gram-negative bacteria are primarily responsible for the serological specificity of the organisms (Wilkinson, 1977) and thus it may be reasonable to assume that these exposed carbohydrates might play a major role in the adherence of *Agrobacterium* to the plant cells. This is in agreement with a previous report from this laboratory that N-acetyl-D-galactosamine and β-D-galactose are involved in adherence during crown gall tumour initiation (Banerjee *et al.*, 1981). However, the ultimate proof that these carbohydrate residues do indeed participate in adherence must come from *in vivo* studies. Results obtained from tumour inhibition studies using various concentrations of carbohydrates for different time period (table 3) demonstrate that N-acetyl-D-galactosamine and β-D-galactose take a positive role in host-bacterium adherence during crown gall tumour initiation.

The minimum time required for tumour inhibition by N-acetyl-D-galactosamine and β-D-galactose at concentrations of 1 mg/ml was 3 h. Lippincott and Lippincott (1980) had reported that cell wall preparations of virulent and avirulent *Agrobacterium* strains could inhibit tumour induction when added only 15 min prior to infection of wound sites of host plants with virulent strains of *Agrobacterium.* Such a short time period for tumour inhibition may be explained by considering that carbohydrate residues when present as part of the bacterial cell wall preparations are afforded a better anchorage than when present free in solution. Thus, free N-acetyl-D-galactosamine and β-D-galactose require a longer period of time to saturate and mask the available binding sites of the host plant cell wall, thereby rendering them inaccessible for further attachment.

On the part of the host, polygalacturonic acid molecules located in outermost region of the plant cell wall and probably exposed by wounding have been shown to participate in the adherence process (Lippincott and Lippincott, 1980).

The mechanism of interaction of N-acetyl-D-galactosamine and β-D-galactose of the *Agrobacterium* cell surface with the plant cell wall polygalacturonic acid molecules is not clear at present and it may be assumed that the interaction takes place either involving van der Wall's forces or by enzymes leading to chemical bonding of the interacting molecules.

Currently we are engaged in studying the physical and chemical properties of *Agrobacterium tumefaciens* LPS in order to get abetter idea of its structure and biological function. Preliminary studies indicate that LPS of *Agrobacterium* are complex molecules comprised of polysaccharide side chain linked to lipid *A via.* a core region containing mainly KDO (2 keto 3 deoxy octulosonate) and orthophosphate. Total carbohydrate content is 20.2%. Fatty acid analyses reveal that hydroxylated fatty acids comprise nearly 60% of the total fatty acids. Physical data indicate that the size of the aggregated particle is in millions of daltons. However, the disaggregated molecular weight worked out to be 250,000 daltons in the analytical ultracentrifuge. Electron microscopy of the LPS by negative staining revealed large vesicle like structures very similar to other bacterial LPS. A protein moiety identified in our LPS preparation suggests, that it is associated with LPS in the native state. Amino acid analysis reveals that it is not an artifact.

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