Restoration of attachment, virulence and nodulation of Agrobacterium tumefaciens chvB mutants by rhicadhesin

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Summary
In contrast to wild-type Agrobacterium tumefaciens strains, \( \beta \)-1,2-glucan-deficient chvB mutants were found to be unable to attach to pea root hair tips. The mutants appeared to produce rhicadhesin, the protein that mediates the first step in attachment of Rhizobiaceae cells to plant root hairs, but the protein was inactive. Both attachment to root hairs and virulence of the chvB mutants could be restored by treatment of the plants with active rhicadhesin, whereas treatment of plants with \( \beta \)-1,2-glucan had no effect on attachment or virulence. Moreover, nodulation ability of a chvB mutant carrying a Sym plasmid could be restored by pretreatment of the host plant with rhicadhesin. Apparently the attachment-minus and virulence phenotype of chvB mutants is caused by lack of active rhicadhesin, rather than directly being caused by a deficiency in \( \beta \)-1,2-glucan synthesis. The results strongly suggest that rhicadhesin is essential for attachment and virulence of A. tumefaciens cells. They also indicate that the mechanisms of binding of Agrobacterium and Rhizobium bacteria to plant target cells are similar, despite differences between these target cells.

Introduction
Attachment of Rhizobium and Agrobacterium bacteria, both members of the Rhizobiaceae family, to host plant cells is an early step in the establishment of the plant-bacterium associations leading to the formation of nitrogen-fixing root nodules and plant tumours, respectively. Attachment of Agrobacterium and Rhizobium cells appears to be a two-step process in which binding to the plant target cell surface is followed by accumulation of the bacteria on the plant cell (Matthysse, 1983; Kijne et al., 1988; Smit et al., 1989b; 1992). The majority of genes that are responsible for tumour induction by Agrobacterium are located on the Ti (tumour-inducing) plasmid (Van Larebeke et al., 1974; Zaenen et al., 1974), whereas most nodulation genes of Rhizobium are localized on the Sym (symbiosis) plasmid (Johnston et al., 1978; Nuti et al., 1979; Hirsch et al., 1980). Introduction of a Ti plasmid into Rhizobium can yield tumorigenic Rhizobium strains, whereas Agrobacterium harbouring a Sym plasmid can nodulate certain leguminous plants (Hooykaas et al., 1977; 1981; 1985). In contrast, genes involved in the attachment of Agrobacterium to cells of host plants are localized on the bacterial chromosome (Douglas et al., 1982; Smit et al., 1987). Similarly, attachment of Rhizobium to the root hairs of host plants is chromosomally encoded (Smit et al., 1986; Kijne et al., 1988).

Cell-to-cell attachment is considered to be a prerequisite for plant tumour formation. Plant molecules involved in the attachment process are poorly characterized. Recently a vitronectin-like protein of plant origin was proposed to play a role in attachment of Agrobacterium (Wagner and Matthysse, 1992). Several bacterial chromosomal loci are reported to be involved in the ability of Agrobacterium tumefaciens to attach to plant cells (Douglas et al., 1982; Cangelosi et al., 1987; Matthysse, 1987; Thomashow et al., 1987). The corresponding mutants are all avirulent. Two of these loci, chvB and exoC, are required for the production of \( \beta \)-1,2-glucan (Puvanesarajah et al., 1985; Cangelosi et al., 1987). chvB mutants do not produce cyclic \( \beta \)-1,2-glucan, since the chvB gene encodes a 235 kDa cytoplasmic membrane protein, that is required for \( \beta \)-1,2-glucan synthesis (Zorreguieta et al., 1988). A third mutant, chvA, is deficient in the excretion of cyclic \( \beta \)-1,2-glucan from the cell (Cangelosi et al., 1989; Iñon de lannino and Ugalde, 1989; O’Connell and Handelsman, 1989). However, the mutant still exports some \( \beta \)-1,2-glucan (O’Connell and Handelsman, 1989) and has been reported to have a leaky phenotype (Van Veen et al., 1987). Since \( \beta \)-1,2-glucan mutants studied so far were found to be attachment deficient, \( \beta \)-1,2-glucan was hypothesized to be necessary for attachment. However, it should be noted that these mutants show a pleiotropic phenotype (Douglas et al., 1985; Puvanesarajah et al., 1985; Cangelosi et al., 1987; Thomashow et al., 1987).

Results from our laboratory showed that binding of Rhizobiaceae to the surface of plant root hairs is mediated by
rhicadhesin, a calcium-binding protein located on the bacterial cell surface (Smit et al., 1989a,b). Since chvB mutants are unable to attach to plant cells, and since it was unknown whether these bacteria produce rhicadhesin, we studied the attachment characteristics of these mutants to pea root hair tips in relation to production of rhicadhesin. Study of chvB mutants was preferred over chvA mutants because of the leaky phenotype of chvA mutants. The results show that the attachment-minus and avirulence phenotypes of chvB mutants result from a lack of active rhicadhesin and not from a lack of β-1,2-glucan. Furthermore, evidence is presented that in the case of nodulation by Sym plasmid-carrying Agrobacterium cells attachment is a prerequisite for infection, being dependent on the presence of active rhicadhesin.

Results

Attachment ability of A. tumefaciens chvB mutants

The chvB mutants grown in TYC and harvested at various growth stages were found to be unable to attach to pea root hair tips, in contrast to the wild-type strain LBA1010, the Ti plasmid-cured strain LBA1251, and Rhizobium leguminosarum 248 (Table 1). This result is consistent with earlier reports on attachment to tobacco and Zinnia cells (Douglas et al., 1982; 1985). The chvB mutants appeared to be affected in the first step of attachment, since the majority of the root hairs were devoid of adhering bacteria.

chvB mutants do not synthesize active rhicadhesin

Cell surface preparations were isolated from A. tumefaciens chvB mutants, the wild-type strain and the Ti plasmid-cured strain, and tested for the presence of rhicadhesin, that is, for its ability to inhibit attachment of A. tumefaciens and R. leguminosarum to pea root hairs. As shown for A. tumefaciens in Table 2, cell surface components isolated from chvB mutants did not possess rhicadhesin activity, whereas cell surface preparations isolated from A. tumefaciens wild-type and plasmid-cured cells did. The same results were obtained with inhibition of attachment of R. leguminosarum 248. This result indicates that no active rhicadhesin is present at the cell surface of chvB mutants. For one chvB mutant strain, A1011, the culture medium as well as a sonicated cell extract were tested for adhesin activity, since it could not be excluded that these mutants still produce rhicadhesin, but are somehow affected in translocation or anchoring of the adhesin. No adhesin activity was found in either fraction (Table 2) indicating that strain A1011 is affected in the production of active rhicadhesin.

In order to check whether the chvB mutant produces rhicadhesin in an inactive form, ME117 bacteria were sheared and the rhicadhesin purification method was followed. By SDS-PAGE, a protein band of about 14 kDa was observed for rhicadhesin purified from strain ME117 (Fig. 1, lane A) at the same position as expected for wild-type rhicadhesin (Fig. 1, lane B) (Smit et al., 1991). Since several concentrations of rhicadhesin isolated from chvB mutants were tested for activity and no inhibition of attachment of wild-type cells was observed (data not shown), it was concluded that ME117 produces rhicadhesin in an inactive form.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>A. tumefaciens</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBA1010</td>
<td>Wild type</td>
<td>12</td>
<td>33</td>
<td>55</td>
</tr>
<tr>
<td>1251</td>
<td>pTi-, pCr-cured</td>
<td>9</td>
<td>25</td>
<td>66</td>
</tr>
<tr>
<td>A1011</td>
<td>chvB</td>
<td>73</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>A1020</td>
<td>chvB</td>
<td>75</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>A1045</td>
<td>chvB</td>
<td>90</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>ME117</td>
<td>chvB</td>
<td>83</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>R. leguminosarum bv. viciae</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>248</td>
<td>Wild type</td>
<td>7</td>
<td>23</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 1. Attachment of Agrobacterium and Rhizobium cells to pea root hair tips.

Table 2. Influence of cell surface preparations, cell extract and culture supernatant from various A. tumefaciens strains on attachment of A. tumefaciens wild-type strain LBA1010 to pea root hair tips.

Addition to standard assay | % attachment in classb
--- | --- | --- |
None | 9 | 31 | 60 |
Cell surface preparation from LBA1010 | 56 | 25 | 19 |
1251 (pTi-, pCr-cured) | 43 | 34 | 23 |
A1011 (chvB) | 12 | 28 | 60 |
A1020 (chvB) | 19 | 28 | 53 |
A1045 (chvB) | 10 | 26 | 64 |
ME117 (chvB) | 5 | 32 | 63 |
Culture supernatant from A1011 (chvB) | 11 | 32 | 57 |
Cell extract from A1011 (chvB) | 14 | 28 | 58 |

a. Additions corresponding to 4 ml bacterial culture were incubated with the pea roots. A. tumefaciens LBA1010 cells were harvested from TY medium, suspended in phosphate buffer and incubated with the roots for 2 h.
b. See Table 1, footnote b.
Role of rhicadhesin in virulence and nodulation

Restoration of attachment ability of chvB mutants by wild-type rhicadhesin

*R. leguminosarum* 248 and *A. tumefaciens* LBA1010 cells, grown under Ca\(^{2+}\)-limiting conditions, are unable to attach to pea root hair tips (Smit *et al.*, 1987; 1991). However, after pre-incubation of pea roots with purified rhicadhesin such bacteria appeared to attach to pea root hairs. This physiological restoration of attachment ability is most likely to be caused by binding of the bacteria to root-bound rhicadhesin (Smit *et al.*, 1991). The results presented in Table 3 show that attachment ability of chvB mutant ME117 could be restored after pretreatment of roots with rhicadhesin, isolated from wild-type *A. tumefaciens*, followed by incubation with mutant cells grown in TY medium, that is, under Ca\(^{2+}\)-limiting conditions. Also rhicadhesin isolated from *R. leguminosarum* bv. vicieae was able to restore attachment (data not shown). However, wild-type levels of attachment, such as that found with restoration of attachment of wild-type *Rhizobium* and *Agrobacterium* grown under Ca\(^{2+}\)-limiting conditions, were not reached. This indicates that binding of rhicadhesin to the cell surface of the chvB mutants is indeed possible, but is less efficient than to wild-type strains of *Agrobacterium* and *Rhizobium*. Restoration of attachment ability was also found when chloramphenicol (50 \(\mu\)g ml\(^{-1}\)) was present during the attachment assay (data not shown). No restoration of attachment ability was found after protease treatment of rhicadhesin (Table 3). Cells of strain ME117 grown in TYC medium did not adhere to the rhicadhesin-pretreated roots (Table 3). Pretreatment of roots with \(\beta\)-1,2-glucan did not result in restoration of attachment ability of ME117 cells (Table 3). The presence of \(\beta\)-1,2-glucan during the incubation with bacteria had no effect on the attachment of ME117 cells; however, this treatment did not inhibit attachment of wild-type strains (data not shown). This result is in agreement with results reported by O’Connel and Handelsman (1989) concerning attachment of chvB mutants to *Zinnia* cells.

Restoration of virulence of *A. tumefaciens* chvB mutants by wild-type rhicadhesin

Virulence of chvB mutants on *Kalanchoë daigremontiana* and *Kalanchoë tubiflora* plants was tested after treatment of plants with purified rhicadhesin, isolated from *A. tumefaciens* or *R. leguminosarum* 248. The chvB mutants, A1011 and ME117, grown in TY or TYC, appeared to be avirulent in the absence of rhicadhesin (less than 1% of the wound sites developed a small tumour) (Fig. 2B). Since attachment ability of chvB mutants could be optimally restored by rhicadhesin after growth of the cells under Ca\(^{2+}\)-limiting conditions, bacteria grown under such conditions were used for testing the possible restoration of virulence. Treatment of *K. daigremontiana* leaf wound sites with rhicadhesin isolated from *A. tumefaciens* LBA1010, prior to inoculation with the chvB mutants, resulted in tumour formation on an average of 50% of the wound sites (Fig. 2E). No tumours were observed when rhicadhesin was added in the absence of bacteria (data not shown). Moreover, no tumours were formed after treatment with rhicadhesin pretreated with protease (Fig. 2C). Addition of \(\beta\)-1,2-glucan to the wound sites prior to inoculation with ME117 did not result in tumour formation (Fig. 2D). This result is in agreement with earlier reports (O’Connel and Handelsman, 1989). Addition of

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**Table 3.** Influence of pretreatment of pea roots with purified rhicadhesin and \(\beta\)-1,2-glucan isolated from wild-type *A. tumefaciens* on attachment of cells of *A. tumefaciens* chvB mutant ME117 to pea root hair tips.

<table>
<thead>
<tr>
<th>Bacterial growth medium (^\text{a})</th>
<th>Root pretreatment</th>
<th>% attachment in class (^\text{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>TY</td>
<td>None</td>
<td>73</td>
</tr>
<tr>
<td>TYC</td>
<td>None</td>
<td>74</td>
</tr>
<tr>
<td>TY</td>
<td>Rhicadhesin</td>
<td>14</td>
</tr>
<tr>
<td>TYC</td>
<td>Rhicadhesin</td>
<td>75</td>
</tr>
<tr>
<td>TY</td>
<td>Protease-treated rhicadhesin (^\text{c})</td>
<td>70</td>
</tr>
<tr>
<td>TY</td>
<td>(\beta)-1,2-glucan</td>
<td>69</td>
</tr>
<tr>
<td>TYC</td>
<td>(\beta)-1,2-glucan</td>
<td>65</td>
</tr>
<tr>
<td>TY</td>
<td>(\beta)-1,2-glucan + rhicadhesin</td>
<td>11</td>
</tr>
</tbody>
</table>

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\(^\text{a}\) Roots were incubated with rhicadhesin (10 ng ml\(^{-1}\)) and/or \(\beta\)-1,2-glucan (0.5 \(\mu\)g ml\(^{-1}\)). The roots were washed in phosphate buffer before incubation with the bacteria. *Agrobacterium* cells were harvested, suspended in phosphate buffer and incubated with the roots for 2 h. See the Experimental procedures section for details.

\(^\text{b}\) TY medium contains (per litre): tryptone (Difco), 5.0 g and yeast extract (Difco), 3.0 g. TYC medium is TY medium supplemented with 7 mM CaCl\(_2\).

\(^\text{c}\) See Table 1, footnote b.

\(^\text{d}\) Rhicadhesin (100 ng ml\(^{-1}\)) was treated with proteinase K (1 mg ml\(^{-1}\)) for 60 min at 37°C prior to incubation with the roots.
Fig. 2. Restoration of virulence of A. tumefaciens chvB mutant strain ME117 on K. dairemonstiana by treatment of the wound sites with rhicadhesin, isolated from A. tumefaciens wild type. Before inoculation with A. tumefaciens wild-type strain LBA1010 (A) or chvB mutant strain ME117 (B–F), wound sites were incubated for 30 min with either buffer (A, B), rhicadhesin treated with proteinase K (C), 10 μg β-1,2-glucan (D), 10 ng rhicadhesin (E) or both β-1,2-glucan and rhicadhesin (F). See the Experimental procedures section for details.

both rhicadhesin and β-1,2-glucan prior to inoculation gave on average a greater number of, and larger tumours than treatment with rhicadhesin only (Fig. 2F). Similar results were obtained when K. tubiflora was used as a test plant (data not shown). The use of rhicadhesin purified from R. leguminosarum strain 248 led to identical results (data not shown).

Restoration of nodulation of A. tumefaciens chvB mutants

Van Veen et al. (1987) showed that, unlike wild-type A. tumefaciens (Hooykaas et al., 1985), chvB mutants harbouring a Sym plasmid of R. leguminosarum bv. phaseoli are not capable of nodulating bean. To investigate if this phenomenon was because of the lack of active rhicadhesin, nodulation of siratro (Macroptillium atropurpureum) by LBA2753 cells grown in TY was initially tested with and without pretreatment of the roots with purified rhicadhesin isolated from strain LBA1010. Siratro was chosen because of its ease as a test system. Wild-type R. leguminosarum bv. phaseoli strain RCC3622 forms nodules on siratro (Fig. 3A). Also A. tumefaciens strain LBA2728, grown in TY or TYC medium, forms nodules on siratro (Fig. 3B). However, chvB strain LBA2753, grown in either TY or TYC medium, does not form nodules on siratro (Fig. 3C). Pretreatment of the roots with rhicadhesin followed by inoculation with LBA2753 cells, grown in TY medium, resulted in the formation of several nodules per plant (Fig. 3D). Incubation of fresh plants, not pretreated with rhicadhesin, with plant medium of the nodulated siratro plants did not result in nodulation. This indicates that: (i) no nodulating wild-type bacteria were present in the medium of the nodulated plants, and (ii) LBA2753 did not permanently regain nodulation ability. Apparently, pretreatment of roots with rhicadhesin is required for nodulation by LBA2753. Similar results were obtained when bean was used as a test plant.

Discussion

chvB mutants of A. tumefaciens were found to be unable
to attach to pea root hairs. According to the model of Smit et al. (1987; 1989b), attachment appeared to be affected in the first step, that is, direct attachment of the bacteria to the surface of the root hair tip. For Rhizobium, this step is non-host-plant-specific and mediated by rhicadhesin. SDS–PAGE of a rhicadhesin preparation of the chvB mutant ME117 yielded a protein band of about 14 kDa running at the same position as wild-type rhicadhesin. However, rhicadhesin preparations isolated from chvB mutants were found to be devoid of adhesin activity. Attachment ability of chvB mutants could be restored by addition of active wild-type rhicadhesin. Presumably chvB mutants produce an inactive form of rhicadhesin. It was concluded that lack of active rhicadhesin is directly responsible for the attachment deficiency of chvB mutants and that other components that are not produced by these mutants do not seem to be directly involved in attachment. Since we were able to isolate rhicadhesin from the cell surface, lack of translocation to the cell surface is not responsible for the attachment deficiency of chvB mutants. The fact that more than one chvB mutant strain has been used and none displays rhicadhesin activity (Table 2) renders the possibility of a (point) mutation in the gene encoding rhicadhesin unlikely. The defect may be caused by improper processing of the rhicadhesin protein or to impaired Ca²⁺ binding. The role of β-1,2-glucan in activation of rhicadhesin will be discussed later. Restoration of attachment ability of chvB mutants by rhicadhesin did not result in wild-type attachment levels, leaving the possibility for requirement of a second efficiency-increasing factor. It is not very likely that this factor is extracellular β-1,2-glucan since addition of β-1,2-glucan did not result in enhancement of attachment, neither in absence or presence of rhicadhesin. Taken together these results are consistent with the hypothesis that, like in the case of Rhizobium, the first step in the attachment of A. tumefaciens is mediated by rhicadhesin. Douglas et al. (1982) reported that chvB mutants are not affected in the synthesis of cellulose fibrils. Our results are consistent with this observation, since accumulation of mutant cells on the plant cell surface (class 3 attachment), a process mediated by cellulose fibrils (Matthysse, 1983; Smit et al., 1987), is observed after treatment of the roots with active rhicadhesin.

Binding of rhicadhesin to Rhizobium and Agrobacterium cells is mediated by Ca²⁺ ions. Only Rhizobiaceae cells grown under Ca²⁺-limiting conditions or grown with calcium and treated with EDTA, were found to be able to bind to rhicadhesin-treated pea roots. Under such growth conditions rhicadhesin bound to bacteria is released into the medium, which probably enables binding of the cells to root-bound adhesin (Smit et al., 1989b; 1991). Accordingly, in order to achieve binding of rhicadhesin to the cell surface of the chvB mutants, the level of Ca²⁺ ions on the cell surface of the mutants had to be low. Furthermore, several phenotypic changes in the cell envelope of chvB mutants could be prevented by growth under calcium-limiting conditions (S. Swart et al., submitted), yielding a more wild-type-like surface for the mutant cells, which may be necessary for anchoring on root-bound rhicadhesin.

Virulence of chvB mutants on K. daigremontiana and K. tubiflora plants was restored by treatment of the wound sites with rhicadhesin prior to inoculation with chvB mutants. On average, the number of tumours was lower in comparison with that induced by the wild-type strain, although the size of the tumours was similar. This result corresponds with the suboptimal restoration of attachment ability found after pretreatment of roots or bacteria with rhicadhesin. Treatment of wound sites with β-1,2-glucan prior to inoculation did not result in tumour formation by chvB mutants, strongly suggesting that restored tumour formation is preceded by restored attachment. Taken together, it can be concluded that lack of active rhicadhesin on the cell surface of the chvB mutants causes the attachment-minus and virulence-minus phenotype of the chvB mutants, rather than lack of β-1,2-glucan, as proposed by Puvanesarajah et al. (1985). However, it is possible that endogenous β-1,2-glucan is necessary for production of active rhicadhesin. The active rhicadhesin used was isolated from a β-1,2-glucan-producing wild-type strain. Under hypo-osmotic conditions β-1,2-glucan may be required for proper membrane assembling, which may be necessary for correct anchoring of rhicadhesin. Beta-1,2-glucan may also be required for stability of rhicadhesin, such as cyclodextrins, that are used for protein stabilization (Szejttl, 1991). This could explain why a greater number, and larger tumours were found in the case where both rhicadhesin and β-1,2-glucan were added in the restoration of virulence. A. tumefaciens chvA mutants still export some of the wild-type amount of β-1,2-glucan (O’Connel and Handelsman, 1989). This may explain why chvA mutants have a leaky phenotype with regard to attachment and rhicadhesin activity (S. Swart, unpublished results) and virulence and nodulation (Van Veen et al., 1987). Dylan et al. (1986) reported homology between the chvA and chvB loci of A. tumefaciens and so-called ndvA and ndvB (for nodulation development) loci in Rhizobium meliloti. In reports on R. meliloti ndvB mutants it is proposed that β-1,2-glucan is not absolutely required for nodule development but might play a role in enhancement of nodulation (Dylan et al., 1990).

Wild-type A. tumefaciens cells harbouring a Sym plasmid of R. leguminosarum bv. phaseoli are able to nodulate siratro and bean whereas chvB mutants harbouring a Sym plasmid cannot (Van Veen et al., 1987; this study). We demonstrated that, only after treatment of the roots of siratro or bean plants with rhicadhesin, strain LBA2753
was able to nodulate these roots. Heterologous complementation of *A. tumefaciens* chvB mutants with *R. meliloti* ndvB genes and vice versa demonstrated the functional interchangeability of the chv and ndv loci (Dylan et al., 1986). Also ndvB mutants of *R. meliloti* were found to be affected in infection (Geremia et al., 1987). However ndvB mutants form pseudonodules whereas chvB mutants harbouring a Sym plasmid do not form nodules at all (Van Veen et al., 1987). Furthermore, ndvB mutants form abortive infection threads and show attachment, although diminished (Dylan et al., 1990). Indeed, we found rhicadhesin activity in cell surface preparations of a ndvB mutant (S. Swart, unpublished results). Apparently, a mutation in the gene encoding the intermediate protein in the β-1,2-glucan synthesis, chvB in the case of *A. tumefaciens* or ndvB in the case of *R. meliloti*, does not result in the same phenotype for these Rhizobiacea. Rhicadhesin activity might be regulated in a different manner in *A. tumefaciens* and *R. meliloti*.

**Comparison of binding properties of Rhizobium and Agrobacterium**

The following evidence supports the hypothesis that *Rhizobium* and *Agrobacterium* bind to the surface of plant cells through a similar mechanism. (i) The chromosome of bacteria from each genus contains the necessary information for establishing an association with host plants of the other genus, provided that the proper plasmid (Sym or Ti) is present (Hooykaas et al., 1977; 1981; 1985; Kondorosi et al., 1982; Hirsch et al., 1985). (ii) The binding properties of cells of both genera are chromosomally encoded (Douglas et al., 1982; Matthysse, 1987; Smit et al., 1986; 1987). (iii) Bacteria from both genera produce rhicadhesin, and, moreover, rhicadhesin, purified from one genus, can inhibit attachment of cells of the other genus to legume root hair tips (Smit et al., 1989b). (iv) For both genera attachment is a two-step process. The second step, mediated by cellulose fibrils, is not essential for the establishment of a successful association (Matthysse, 1983; Smit et al., 1987). The first step, mediated by rhicadhesin, is necessary for an interaction between plant and bacterium (Smit et al., 1989b; this paper). (v) The attachment ability to root hairs as well as virulence of chvB mutants can be restored by addition of rhicadhesin isolated from either *Rhizobium* or *Agrobacterium* (this paper). (vi) The virulence of chvB mutants on *Kalanchoë* as well as nodulation of bean and siratro by chvB mutants harbouring a Sym plasmid of *R. leguminosarum* bv. phaseoli can be restored by pretreatment of the roots with rhicadhesin (this paper).

**Experimental procedures**

**Bacterial strains and culture conditions**

Bacterial strains of *A. tumefaciens* and *R. leguminosarum* used are listed in Table 4. Compositions of the media A+ and LC (Smit et al., 1987) have been described previously. TY medium contains (per litre): tryptone (Difco), 5.0 g and yeast extract (Difco), 3.0 g. TYC medium is TY medium supplemented with 7 mM CaCl₂. *Rhizobium* and *Agrobacterium* species were maintained on solid A+ and solid LC medium, respectively. For attachment assays, bacteria were cultivated on a rotary shaker (180 r.p.m.) at 28°C in 100 ml Erlenmeyer flasks, containing 50 ml TYC medium. For purification of rhicadhesin, bacteria were grown in either 500 ml or 21 Erlenmeyer flasks containing 250 ml or 1.25 l TYC medium, respectively.

**Plants and plant culture conditions**

Seeds of pea (*Pisum sativum* cv. finale; Cebeco) were surface sterilized and cultivated as described previously (Smit et al., 1986). Seeds of siratro (*Macroptilium atropurpureum*), a generous gift from Dr B. G. Rolfe, Australian National University, Canberra, Australia) were surface sterilized as described for pea seeds and germinated on solid Jensen agar (Vincent, 1970) at 28°C for about 20 h. Subsequently, plants were grown in liquid Jensen medium at 28°C in a growth chamber. The light intensity at table surface was 20 000 lux during a 16 h light period. *K. daigremontiana* and *K. tubiflora* plants were cultivated as described by Ooms et al. (1980). Bean seeds (*Phaseolus vulgaris* cv. prelude) were surface sterilized and germinated between wetted Whatman filter paper at 22°C. After germination for 4 d the seedlings were grown in glass tubes on solid Jensen medium slopes at 22°C in a growth chamber with light intensity of 20 000 lux during a 16 h light period.

**Table 4. Bacterial strains.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or phenotype</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
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<td>LBA1010</td>
<td><em>A. tumefaciens</em> C58 chromosome, pTiB6</td>
<td>Koekman et al. (1982)</td>
</tr>
<tr>
<td>LBA1251</td>
<td><em>A. tumefaciens</em> C58 chromosome, pTi and pC-cured</td>
<td>Rosenberg and Huguet (1984)</td>
</tr>
<tr>
<td>A1011</td>
<td><em>A. tumefaciens</em> C58 chromosome chvB::Tn5</td>
<td>Douglas et al. (1982)</td>
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<td>A1020</td>
<td><em>A. tumefaciens</em> C58 chromosome chvB::Tn5</td>
<td>Douglas et al. (1982)</td>
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<td><em>A. tumefaciens</em> C58 chromosome chvB::Tn3::HoHo1</td>
<td>Douglas et al. (1985)</td>
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<td>LBA2728</td>
<td><em>A. tumefaciens</em> LBA1010 pSym9 of RCC3622</td>
<td>Van Veen et al. (1987)</td>
</tr>
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<td>LBA2753</td>
<td><em>A. tumefaciens</em> ME117, pSym9 of RCC3622</td>
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<td>RCC3622</td>
<td><em>R. leguminosarum</em> bv. phaseoli (wild-type)</td>
<td>Hooykaas et al. (1985)</td>
</tr>
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<td>248</td>
<td><em>R. leguminosarum</em> bv. viciea (wild-type)</td>
<td>Josey et al. (1979)</td>
</tr>
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</table>
Attachment assay

The attachment assay used has been described previously by Smit et al. (1986). Briefly, roots were immersed in a suspension of bacteria, washed 10 times and attachment was quantified by randomly screening at least 100 developing root hairs with a phase-contrast microscope. Attachment was classified in three classes: class 1, no attached bacteria; class 2, few bacteria, attached to the root hair; class 3, many attached bacteria, forming two or more layers of bacteria or a cap-like aggregate at the tip of the root hair (cap formation). The percentage of root hairs of each class was calculated. The variability of the test is about 10% and depends largely on the condition of the roots.

Purification of rhicadhesin

Procedures for the purification of rhizobial rhicadhesin (Smit et al., 1989b), isolation of cell surface preparations from A. tumefaciens strains (Smit et al., 1989a), and testing the presence of rhicadhesin in culture supernatants of cells (Smit et al., 1991) have been described previously. Presence of rhicadhesin inside bacteria was examined after sonication of the cells for 30 s in a sonifier cell disrupter (Branson Sonic Power Comp.). After sonication of the cells, the suspension was clarified by ultracentrifugation (100 000 × g for 2 h) and tested for adhesin activity.

SDS–PAGE and protein staining

SDS–PAGE was performed as described by Schägger and Von Jagow (1987). Proteins were silver-stained according to Oakley et al. (1980). The molecular masses of the marker proteins were: bovine serum albumin, 66 kDa; egg albumin, 45 kDa; carbonic anhydrase, 29 kDa; β-lactoglobulin, 18 kDa; and lysozyme, 14 kDa.

Isolation of β-1,2-glucan

Stationary cells of A. tumefaciens LBA1010, grown in TYC, were harvested by centrifugation at 10 000 × g for 10 min. Pellets were extracted with 1% trichloroacetic acid (TCA) for 30 min at room temperature. TCA extracts were neutralized, concentrated, chromatographed on Sephadex G-50 (Pharmacia), and tested according to the method of Miller et al. (1986).

Test for rhicadhesin activity

Activity of rhicadhesin was determined by measuring the ability to inhibit attachment of LBA1010 cells to pea root hair tips. To test whether a fraction contained active rhicadhesin, two lateral pea root tips were wounded with 0.1 μg/ml rhicadhesin harvested from A. tumefaciens and tested for adhesin activity.

Virulence assays

In the greenhouse K. daigremontiana and K. tubiflora plants were wounded on leaves and stems, respectively. A solution of rhicadhesin (2–10 ng per wound site) and/or β-1,2-glucan (10 μg per wound site) in 25 mM phosphate buffer, pH 7.5, was added to a volume of 5–25 μl per wound site. Controls were incubated with phosphate buffer only. Ten to twenty microlitres of A. tumefaciens chvB mutant cells, grown in TY medium, harvested and suspended in phosphate buffer to an A600 value of 1.0, were added to each wound site 30 min after incubation with rhicadhesin or buffer. As a control wild-type A. tumefaciens was added to wound sites. Tumour formation was quantified three weeks after inoculation.

Nodulation assay

After germination, sirato seedlings were transferred to Jensen medium (5–6 seedlings per glass tube containing 25 ml medium) and grown overnight at 28°C in the dark. Subsequently, an appropriate number of roots were treated with 0.1 μg/ml rhicadhesin in Jensen medium for 30 min. Twenty-five microlitres of A. tumefaciens LBA2753 cells, grown in TY medium, harvested and resuspended in Jensen medium to an A600 value of 0.1, were added to each tube. Strains LBA2728 and RCC3622 were used as controls. Nodulation was quantified 3 weeks after inoculation.

After germination, bean seedlings were grown on Jensen medium for 3 d in the dark before inoculation. One millilitre 25 mM phosphate buffer, pH 7.5, or 1.0 ml rhicadhesin (0.5 μg/ml) in phosphate buffer was added to the roots and after 1 h the roots were inoculated with 3.0 ml of A. tumefaciens strain LBA2753 cells, grown in TY medium, harvested and resuspended in Jensen medium to an A600 value of 0.05. Strains LBA2728 and RCC3622 were used as controls. Nodulation was quantified 5 weeks after inoculation.

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References


