Roots induced on cucumber cotyledons by the agropine Ri plasmid TR-DNA exhibit the transformed phenotype

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ABSTRACT
Cucumber explants were transformed by Agrobacterium strains carrying Ri plasmids with functional TL and TR-DNAs, and by strains whose pRi had an intact TR-DNA but a disarmed TL-DNA lacking open reading frames (ORFs) 3 to 9, 10 (rol A), 11 (rol B), 12 (rol C), 13, 14, 15 (rol D), 16 and 17. Roots induced by all strains exhibited extensive root hair formation under axenic conditions, synthesised opines, and contained TR-specific DNA. These results confirm that the TR-DNA of an agropine Ri plasmid is able to elicit the transformed root phenotype in this plant.

INTRODUCTION
Roots of higher plants transformed by strains of Agrobacterium rhizogenes carrying wild-type agropine Ri plasmids exhibit characteristic traits in vitro. Such roots are highly branched with rapid, negatively geotropic and/or plagiotropic growth (Spané et al. 1981; Tepper and Tempé 1982; Bilby et al. 1987; Cardarelli et al. 1987). In general, the production of Ri transformed roots correlates with the presence of the agropine Ri TL-DNA (Vilaine and Cassée-Delbart 1987). Cardarelli et al. (1987b) have suggested that during the induction of transformed roots, the tms genes of the agropine pRi TR-DNA encoding the enzymes involved in auxin synthesis, trigger the differentiation of cells containing auxin-responsive agropine pRi TL-DNA. These authors propose that the TR-DNA is not essential for transformed root induction, as endogenous plant auxins can substitute for indoleacetic acid encoded by the TR-DNA tms genes.

In the present report, the effect has been investigated of the agropine pRi TR-DNA on the induction of roots in cucumber. These organs incited by Agrobacterium strains carrying an unmodified agropine Ri TR-DNA, but a disarmed TL-DNA lacking the rol A, B, C and D genes, exhibited characteristics typical of Ri plasmid transformed roots.

MATERIALS AND METHODS
Seeds of Cucumis sativus cv Rebella (Rijk Zwaan, Burg. Crezeelaan 40, De Lier, Holland) were surface sterilised in 10% v/v "Domestos" bleach (30 min), followed by 3 washes with sterile water. Seeds were germinated on agar-solidified (0.8% w/v; Sigma) hormone-free Murashige and Skoog (1962) medium (MS) at 25°C under daylight fluorescent illumination (1.6 W m⁻²; Davey et al. 1989).

Cotyledons were excised from 7 d old seedlings and scored across the midrib with a scalpel in an overnight culture of Agrobacterium rhizogenes. The bacterial strains used were A4T (TL⁻TR⁻), A4T::Neo (TL⁻TR⁺), BN1010 (TL⁻TR⁻) and BN1010: :Neo (TL⁻TR⁺). A4T: :Neo was also designated A4TII (McInnes et al. 1987). A4T was a Cm⁻Tc⁻ Rif⁻ derivative of A. tumefaciens strain C58 with pTiC58 replaced by pRlA4 (Moore et al. 1979). Strain A4T: :Neo carried pRlA4::pAMNeo10, which had the nop. neo· gene cotransformed into HindIII fragment 11 of the pRlA4 TL-DNA (McInnes et al. 1987). Plasmid BN1010, carried by strain BN1010, was obtained by replacement mutagenesis of ORFs 3 to 9, 10 (rol A), 11 (rol B), 12 (rol C), 13, 14, 15 (rol D), 16 and 17 of pRlA4 following mobilisation of pBW101 into Agrobacterium strain A4T (McInnes et al. 1989). Plasmid BN101 consisted of a 4.2 kb EcoRI/CleII fragment of pBR325 placed between regions of HindIII fragments 16 and 11 of pFW94 (Huffman et al. 1984). The ends of these Rl homology regions were separated by the Tn5 kanamycin resistance gene from pN60 (Pharmacia Ltd.). Strain BN1010::Neo had the same nop. neo· gene as strain A4T: :Neo, cotransformed into the disarmed TL-DNA (lacking rol genes A, B, C and D together with ORFs 3 to 9, 13, 14, 16 and 17) of BN1010 (McInnes et al. 1989).

More details of the construction of pRlA4::pAMNeo10, pBW1010 and pBW1010::Neo, together with the maintenance of bacterial strains carrying these plasmids, are given by Morgan et al. (1987) and McInnes et al. (1989).
Control inoculations were performed by scoring the cotyledons with a scalpel dipped in bacterial culture medium. The excised cotyledons were cultured on 15 ml aliquots of agar-solidified MSO medium with 500 µg ml⁻¹ of cefotaxime, contained in 9 cm diameter Petri dishes. The inoculation response was recorded after 28 d incubation at 27°C (1.0 Wm⁻²; daylight fluorescent illumination).

Roots were excised from cotyledons and root tips (1-cm lengths) cultured on agar-solidified MSO medium containing 500 µg ml⁻¹ of cefotaxime for the first two, 21 day passages. Thereafter, the antibiotic was omitted from the culture medium. The maintenance of cloned roots, opine analysis and the confirmation of agropine Ri T-DNA integration into the plant genome, were performed as described previously (McInnes et al. 1989). Fresh weight determinations were made on cultured roots during their second passage on MSO agar medium.

RESULTS

Inoculation of cucumber cotyledons with strains A4T, A4T:Neo, BN1010 and BN1010:Neo, induced the production of profusely branched roots at the inoculation sites (Fig 1a,b). All Ri-transformed roots were branched, and showed rapid plagiotropic growth in vitro on hormone-free MSO agar medium following excision from the seedling cotyledons (Fig 1c). Such roots exhibited extensive root hair development (Fig 1d); some clones also produced callus under culture conditions. Cotyledons which were not inoculated with bacteria produced some roots when wounded, but these were few in number. Non-transformed cucumber roots exhibited poor, necrotic growth under the same culture conditions as Agrobacterium-induced roots.

The growth of roots induced by strains A4T, A4T:Neo, BN1010 and BN1010:Neo was comparable after 10 and 20 days of culture on MSO agar medium (Table 1). In contrast, non-transformed cucumber roots produced less biomass under the same conditions.

Paper electrophoresis of crude tissue extracts showed cloned roots transformed by the four Agrobacterium strains to synthesise silver nitrate positive compounds, which ran to similar positions as authentic agropine and mannopine + mannopinic acid (Fig 2). All plant extracts contained a silver nitrate staining compound which obscured the detection of agrocinic acid in transformed roots.

HindIII digestion of plant DNA, followed by Southern blot hybridisation to the pR1 TR-DNA probe pFW41 (Huffman et al. 1984), confirmed the presence of the TR-DNA in cloned roots transformed by the TL-TR+ Agrobacterium strain BN1010 (Fig 3a). TR-DNA was also detected in roots transformed by strains A4T and A4T:Neo.

<table>
<thead>
<tr>
<th>Bacterial strain used for root Induction</th>
<th>10 d</th>
<th>20 d</th>
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<tbody>
<tr>
<td>A4T</td>
<td>454 ± 20</td>
<td>2051 ± 49</td>
</tr>
<tr>
<td>A4T:Neo</td>
<td>442 ± 31</td>
<td>2074 ± 36</td>
</tr>
<tr>
<td>BN1010</td>
<td>460 ± 26</td>
<td>2105 ± 52</td>
</tr>
<tr>
<td>BN1010:Neo</td>
<td>450 ± 18</td>
<td>2042 ± 41</td>
</tr>
<tr>
<td>Non-transformed roots</td>
<td>72 ± 11</td>
<td>85 ± 13</td>
</tr>
</tbody>
</table>

| TABLE 1 Growth of transformed cucumber roots (mg fresh weight) measured after 10 and 20 days during the third passage on MSO agar medium. Values are for 5 replicates ± standard error. Weight of starting material/replicate = 50 ± 10 mg. |

The presence of an intact TL-DNA in a A4T transformed root clone and a disarmed TL-DNA in a BN1010-transformed root clone, was also confirmed by Southern blot hybridisation to the pR1 TL-DNA probe pFW04 (Huffman et al. 1984; Fig 3b).

Roots transformed by A4T:Neo and BN1010:Neo expressed resistance to kanamycin concentrations of 100 µg ml⁻¹ when cultured on medium containing the antibiotic. In contrast, non-transformed cucumber roots, and those transformed by strains A4T and BN1010, were inhibited in their growth by kanamycin concentrations in excess of 50 µg ml⁻¹.

DISCUSSION

Inoculation of cucumber cotyledons with A. rhizogenes strains BN1010 (TL-TR+) and BN1010:Neo (TL-TR–) induced roots which were morphologically similar to those roots resulting from transformation by A. rhizogenes strain A4T, carrying the wild-type pR1A4 (TL-TR+), and by strain A4T:Neo. The latter strain also had full length TL and TR-DNAs (TL-TR+), with, in addition, the nap neo– gene cotransferred into the TL-DNA. Transformed cucumber roots were also similar phenotypically to roots of plants such as Nicotiana tabacum and Solanum nigrum transformed by Agrobacterium strains A4T and A4T:Neo (Davey et al. 1987). Opine analysis indicated the expression of TR-DNA genes in transformed cucumber roots.

Southern blot analysis confirmed the presence of TR-specific DNA in roots transformed by Agrobacterium strain BN1010 (TL-TR+). The only regions of the original TL-DNA of pR1A4 which remained on pBN1010 were a BamHI-SalI fragment of HindIII fragment 11 and an EcoRI-HindIII fragment of HindIII fragment 10 (McInnes et al. 1989). The BamHI-SalI fragment

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