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VirD4-independent transformation by CloDF13 evidences an unknown factor required for the genetic colonization of plants via *Agrobacterium*

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**Introduction**

The natural trans-kingdom genetic transfer from *Agrobacterium* tumefaciens to plants during tumourigenesis represents a sophisticated process of bacterial colonization (Hooykaas and Beijersbergen, 1994; Hansen and Chilton, 1999; Zupan et al., 2000). Such an infection relies on transfer of a precise DNA fragment (the T-DNA) flanked by two 25 bp directly repeated sequences (the T-DNA borders). The T region is part of the large bacterial tumour-inducing plasmid (the pTi) and is exported as a DNA/protein complex (the T-complex) from the bacterial cell to the plant cell by the cognate virulence (vir) genes. Eventually the T-DNA integrates into the plant genome and expresses a number of oncogenic (onc) genes resulting in tumourous overgrowth, the so-called crown-gall disease. More recently, it was found that *Agrobacterium* can mediate DNA transfer also to the yeast *Saccharomyces cerevisiae* (Bundock et al., 1995) and filamentous fungi (De Groot et al., 1998). The genetic requirements for T-DNA transfer to plants have been extensively studied. The bacteria detect plant metabolites, such as acetosyringone (AS), via the VirA chemoreceptor. Then the activated transcription factor VirG triggers the transcriptional activation of the remaining vir genes but the T-region (the so-called crown-gall disease). More recently, it was found that *Agrobacterium* can mediate DNA transfer also to the yeast *Saccharomyces cerevisiae* (Bundock et al., 1995) and filamentous fungi (De Groot et al., 1998).

The genetic requirements for T-DNA transfer to plants have been extensively studied. The bacteria detect plant metabolites, such as acetosyringone (AS), via the VirA chemoreceptor. Then the activated transcription factor VirG triggers the transcriptional activation of the remaining vir genes but the T-region (the so-called crown-gall disease). More recently, it was found that *Agrobacterium* can mediate DNA transfer also to the yeast *Saccharomyces cerevisiae* (Bundock et al., 1995) and filamentous fungi (De Groot et al., 1998).

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**Summary**

*Agrobacterium* uses a mechanism similar to conjugation for trans-kingdom transfer of its oncogenic T-DNA. A defined VirB/VirD4 Type IV secretion system is responsible for such a genetic transfer. In addition, certain virulence proteins as VirE2 can be mobilized into host cells by the same apparatus. VirE2 is essential to achieve plant but not yeast transformation. We found that the limited host range plasmid CloDF13 can be recruited by the virulence apparatus of *Agrobacterium* for transfer to eukaryotic hosts. As expected the VirB transport complex was required for such trans-kingdom DNA transfer. However, unexpectedly, the coupling factor VirD4 turned out to be necessary for transfer to plants but not for transport into yeast. The CloDF13 encoded coupling factor (Mob) was essential for transfer to both plants and yeast though. This is interpreted by the different specificities of Mob and VirD4. Hence, Mob being required for the transport of the CloDF13 transferred DNA (to both plants and yeast) and VirD4 being required for transport of virulence proteins such as VirE2. Nevertheless, the presence of the VirE2 protein in the host plant was not sufficient to restore the deficiency for VirD4 in the transforming bacteria. We propose that Mob functions encoded by the plasmid CloDF13 are sufficient for DNA mobilization to eukaryotic cells but that the VirD4-mediated pathway is essential to achieve DNA nuclear establishment specifically in plants. This suggests that other *Agrobacterium* virulence proteins besides VirE2 are translocated and essential for plant transformation.

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evidences support a model in which the VirE2 or VirF proteins are not compulsory implicated in the transport of the T-complex through the bacterial envelope but rather have a function in the plant cell. In fact, it has recently been shown that both VirE2 and VirF proteins share the peculiarity of being transferred independently of the T-strand by the Agrobacterium system to the plant cell (Vergunst et al., 2000).

It has been proposed that export of the T-complex from the Agrobacterium cell occurs via a mechanism that resembles bacterial conjugation. Conjugative plasmids encode sets of genes responsible for two distinct steps. First, mobilization (mob) genes, which encode a relaxase and auxiliary proteins to nick the DNA at a specific site in the origin of transfer (oriT) sequence to form the so-called relaxosome. From this relaxosome a single-stranded DNA is released and introduced into the recipient cell (Lanka and Wilkins, 1995). Second, mating-pair formation (mpf) genes, which encode proteins that form a multicomponent mating-complex that allows the single-stranded DNA to be transported from donor to recipient cell. Although a correlation between plius production and the presence of such mpf genes has been reported, there is no clarity yet about the role of the conjugative plius in the DNA-transfer process. In addition to mob and mpf, there is a third class of genes (e.g. traG of the IncP plasmid RP4 and virD4 of the Ti plasmid) encoding what has been proposed as ‘coupling proteins’ (Cabezón et al., 1997) which may act as ‘molecular motors’ mediating the transport of the nucleoprotein complex from the relaxosome to the VirB-like transport structure (see de la Cruz and Lanka, 1998 and references therein). TraG-like proteins share homology around two putative nucleotide-binding motifs and are essential elements among DNA transfer systems. The similarities between T-DNA transfer and bacterial conjugation have become clearer during the last few years. Specifically, the Ti plasmid virulence machinery can mediate the transfer not only of T-DNA but also of the IncQ plasmid RSF1010 either to plant cells (Buchanan-Wollaston et al., 1987) or between agrobacteria (Beijersbergen et al., 1992). This DNA transfer has been shown to depend particularly on a functional virB operon and virD4. Ten of the 11 virB genes have been shown to be essential for tumourigenesis (Berger and Christie, 1994) and their products have been described as to be associated with the bacterial envelope, either the inner or the outer membrane (Beijersbergen et al., 1994). Additionally, it has been shown that the VirB products are responsible for the appearance of a 10 micron diameter T-pilus at the surface of Agrobacterium (Fullner et al., 1996; Lai and Kado, 1998).

On the other hand, the presence of an IncQ (RSF1010-derivative) plasmid has been shown to inhibit tumour formation in plants, an effect that could be overcome by overexpressing virB9, virB10 and virB11 (Ward et al., 1991) and appeared to be related to the ability of agrobacteria to serve as VirE2 donor cell in extracellular complementation experiments (Binns et al., 1995). Hence, it has been proposed that the IncQ and the T-complex compete for the VirB-made transport apparatus. Expression of the osa gene of the IncW plasmid pSa can also suppress tumourigenesis incited by A. tumefaciens probably by inhibiting the export of VirE2 (Lee et al., 1999). Inhibition of tumour formation by osa could not be alleviated by overexpression of virB genes.

In our study, we were interested in the bacteriocinogenic CloDF13 plasmid because its mobilization was shown to be independent of the F plasmid traD gene (Van de Pol et al., 1978), a trag-like coupling factor. Recently, it was reported that CloDF13 can also be mobilized by the mpf genes of plasmid R388 (Nuñez and de la Cruz, 2001). CloDF13 originates from Enterobacter cloacae (Tieze et al., 1969) and belongs to a group of mobilizable, although non-self-transmissible plasmids, with a small size that can be maintained at high copy number in bacteria (Veltkamp and Stuijle, 1981). Hence, we wanted to investigate whether particular CloDF13 conjugal functions could substitute or interact with certain components of the Agrobacterium virulence system and arbitrate DNA transfer to eukaryotic cells. Here, we provide evidence that the mob-oriT region of the plasmid CloDF13 is responsible for its cognate DNA transfer from Agrobacterium in an efficient manner. Our results lead us to suggest that CloDF13 transfer to eukaryotic cells solely relies on a functional virB operon. In this CloDF13 transfer system VirD4 and virE2 appeared as specific genetic requirements for transfer to plants and dispensable to yeast. Interestingly, both virD4 and virE2 mutations could be complemented by coinoculating tobacco plantlets with wild-type vir bacteria.

Results

Trans-kingdom mobilization of a CloDF13 chimeric plasmid by Agrobacterium requires vir-gene activation, low temperature and long mating time

The CloDF13 plasmid is a small, non-conjugative plasmid which can be mobilized among E. coli cells by several conjugative plasmids (e.g. F(IncF), R46(IncN), RP4 (IncP) and R388 (IncW); Cabezón et al., 1997). The mobilization genes of CloDF13 are distinctly different from those of other mobilizable plasmids, such as the IncQ plasmid RSF1010. In addition, CloDF13 seems to encode a protein related to the family of coupling proteins, such as pTi VirD4 and RP4 TraG. Hence, we were interested to find out whether CloDF13 could be mobilized by the pTi virulence system in interkingdom crosses and which Vir-proteins would be required for such a DNA transfer.
Initially, we chose yeast (Saccharomyces cerevisiae) as recipient-cell species because of experimental convenience. Therefore, we constructed the plasmid pClo-LEU (Fig. 1), containing the mobilization region of CloDF13, the RK2 replicator for maintenance in agrobacteria, the yeast leu2 selection gene and the yeast 2μ replicator. We then did mating experiments between A. tumefaciens and S. cerevisiae RSY12, which is a haploid leu2 mutant, to test for plasmid transfer from bacteria to yeast. Indeed transfer of pClo-LEU could be detected with a wild-type vir bacterial strain (LBA1100).

To study the mechanism of transfer of the pClo-LEU plasmid from agrobacteria to yeast, we assayed different values of four important parameters for Agrobacterium-mediated DNA transfer during our bacteria/yeast co-cultivation. Namely: (i) acidity of the medium (pH 5.3 versus pH 7); (ii) temperature (23°C versus 33°C); (iii) mating time (20 h, 40 h and 60 h); and (iv) presence or absence of the vir-gene inducer acetosyringone (AS). The results from Table 1 show that an acidic medium (pH 5.3), a low temperature (23°C) and the presence of the vir-gene inducer acetosyringone (AS) were essential during the bacteria/yeast co-cultivation for the recovery of Leu+ transformed yeast colonies. The duration of mating time was also critical because high numbers of Leu+ yeast colonies were only observed after a co-cultivation time of at least 60 h. Then the estimated pClo-LEU transfer frequency from the bacterial strain LBA1100 was ~ 10⁻⁶. This value decreased one order of magnitude per 20 h shortening in mating time. A similar duration of co-cultivation was also necessary for T-DNA transfer from agrobacteria to yeast (data not shown). Hence, we concluded that pClo-LEU was mobilized to yeast via a typical process mediated by the Agrobacterium virulence system. For that, transcriptional activation of the vir regulon is necessary and the particular mating complex in the agrobacterial donor, responsible for DNA/protein translocation, needs to be functionally established.

**Role of Vir-proteins in the interkingdom CloDF13 mobilization**

To establish which of the Vir-proteins were involved in the interkingdom transfer of CloDF13, we tested several vir-mutants for their ability to mobilize pClo-LEU to yeast. As it is shown in Table 2, transfer did occur when wild-type...
vir bacterial strains (strains LBA1010, LBA1100 and GV3101) were used. However, lack of vir genes (strain LBA288) resulted in no plasmid transfer. The virA (LBA1142), virB operon (LBA1143) and virG (LBA1145) genes were essential for transfer to occur. This was expected as the VirA and VirG proteins are regulators of the expression of the vir-regulon and it is believed that VirB proteins determine the mating structure. Mutants impaired in the gene for the single stranded DNA binding protein VirE2 (strains LBA1149 and ATvirE2) showed a tenfold lower frequency of transfer, in accordance with T-DNA transfer assays to yeast (data not shown). The host-range gene virF (strain LBA2561) was not necessary for CloDF13 mobilization. As the CloDF13 mobilization region determines its own oriT sequence and the cognate relaxase protein, we expected that the pTi-encoded specific relaxase VirD2 would not be necessary for CloDF13 transfer. This was indeed the case: strains with a mutation in virD2 (LBA1147) or complete deletion of virD2 (ATvirD2) were equally mobilization-proficient as the wild-type strains. Similarly, CloDF13 transfer from Agrobacterium to yeast could be accomplished from bacteria with a mutation in the gene coding for the coupling factor VirD4 (strain LBA1148). As VirD4 is essential for T-DNA transfer to yeast, it is probable that the protein encoded by the CloDF13 mobilization region (MobB, which resembles VirD4) can take over its function and facilitate pClo-LEU transfer. To confirm that such a characteristic is intrinsic for CloDF13-like plasmids, we also assayed a RSF1010 (IncQ) derivative plasmid. RSF1010 could not be transferred from yeast to the mutated virD4 bacterial strain LBA1148 (data not shown).

The CloDF13 mobB and mobC genes are essential for trans-kingdom transfer

We constructed the plasmid pClo-LEU by inserting the mobilization region from CloDF13 into a wide host range replicon (see Fig. 1). As a control, we created the plasmid pEC-LEU, which is identical to pClo-LEU but devoid of CloDF13 sequences. As expected, pEC-LEU could not be transferred from wild-type agrobacteria to yeast cells (see Table 3). In order to analyse the CloDF13 genetic elements that were required for the observed plasmid transfer, we expected the different CloDF13 genetic components in the transfer efficiency of CloDF13-derivative plasmids from agrobacteria to yeast.

### Table 2. Estimated transfer of pClo-LEU from different *A. tumefaciens* donor strains to the *S. cerevisiae* recipient strain RSY12.

<table>
<thead>
<tr>
<th>Bacterial strain (vir mutation)</th>
<th>No. of RSY12 colonies (×10⁶)</th>
<th>No. Leu/RSY12 colonies</th>
<th>Frequency of Leu⁺ per recipient cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBA288 (No vir)</td>
<td>2.2</td>
<td>0</td>
<td>&lt;2.2 × 10⁻⁴</td>
</tr>
<tr>
<td>LBA1010</td>
<td>2.3</td>
<td>1175</td>
<td>0.5 × 10⁻⁵</td>
</tr>
<tr>
<td>LBA1100</td>
<td>2.1</td>
<td>1200</td>
<td>0.5 × 10⁻⁵</td>
</tr>
<tr>
<td>GV3101</td>
<td>1.3</td>
<td>2700</td>
<td>2.0 × 10⁻⁵</td>
</tr>
<tr>
<td>LBA1142 (virA)</td>
<td>1.6</td>
<td>0</td>
<td>&lt;1.6 × 10⁻⁴</td>
</tr>
<tr>
<td>LBA1143 (virB)</td>
<td>1.4</td>
<td>0</td>
<td>&lt;1.4 × 10⁻⁴</td>
</tr>
<tr>
<td>LBA1145 (virG)</td>
<td>1.2</td>
<td>0</td>
<td>&lt;1.2 × 10⁻⁴</td>
</tr>
<tr>
<td>LBA1147 (virD2)</td>
<td>1.5</td>
<td>2500</td>
<td>1.6 × 10⁻⁵</td>
</tr>
<tr>
<td>ATvirD2 (virD2)</td>
<td>1.4</td>
<td>2475</td>
<td>1.7 × 10⁻⁵</td>
</tr>
<tr>
<td>LBA1148 (virD4)</td>
<td>1.1</td>
<td>1400</td>
<td>1.4 × 10⁻⁵</td>
</tr>
<tr>
<td>LBA1149 (virE2)</td>
<td>2.0</td>
<td>154</td>
<td>0.7 × 10⁻⁴</td>
</tr>
<tr>
<td>ATvirE2 (virE2)</td>
<td>2.3</td>
<td>160</td>
<td>0.7 × 10⁻⁵</td>
</tr>
<tr>
<td>LBA2561 (virF)</td>
<td>1.5</td>
<td>1250</td>
<td>0.8 × 10⁻⁵</td>
</tr>
</tbody>
</table>

a. The bacterial strains LBA1010, LBA1100 and GV3101 all contain a wild-type vir set of genes in their respective pTi plasmid. The bacterial strain LBA288 is a LBA1010 derivative in which the pTi plasmid was cured. All bacterial strains contained the plasmid pClo-LEU and matings were performed at 23°C, in pH 5.3 medium containing 0.2 mM acetosyringone as described (see Experimental procedures).

b. Counts are values per milliliter of mating mixture.

### Table 3. Effect of the different CloDF13 genetic components in the transfer efficiency of CloDF13-derivative plasmids from agrobacteria to yeast.

<table>
<thead>
<tr>
<th>Line</th>
<th>Bacterial strain</th>
<th>No. of RSY12 colonies (×10⁶)</th>
<th>No. Leu/RSY12 colonies</th>
<th>Frequency of Leu⁺ per recipient cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LBA1100 [pClo-LEU]</td>
<td>2.3</td>
<td>1350</td>
<td>0.6 × 10⁻⁵</td>
</tr>
<tr>
<td>2</td>
<td>LBA1100 [pClo-LEU]</td>
<td>1.8</td>
<td>0</td>
<td>&lt;1.8 × 10⁻⁴</td>
</tr>
<tr>
<td>3</td>
<td>LBA1100 [pClo<del>E</del>E<del>L</del>E<del>U</del>]</td>
<td>3.0</td>
<td>360</td>
<td>1.2 × 10⁻⁵</td>
</tr>
<tr>
<td>4</td>
<td>LBA1100 [pClo<del>E</del>H<del>L</del>E<del>U</del>]</td>
<td>1.8</td>
<td>3200</td>
<td>1.7 × 10⁻⁵</td>
</tr>
<tr>
<td>5</td>
<td>LBA1100 [pClo<del>E</del>B<del>E</del>L<del>E</del>U~]</td>
<td>3.0</td>
<td>0</td>
<td>&lt;3.0 × 10⁻⁵</td>
</tr>
<tr>
<td>6</td>
<td>LBA1100 [pClo<del>E</del>C<del>E</del>L<del>E</del>U~]</td>
<td>3.2</td>
<td>0</td>
<td>&lt;3.2 × 10⁻⁵</td>
</tr>
<tr>
<td>7</td>
<td>LBA1148 [pClo<del>E</del>L<del>E</del>U~]</td>
<td>3.0</td>
<td>400</td>
<td>1.3 × 10⁻⁵</td>
</tr>
<tr>
<td>8</td>
<td>LBA1148 [pClo<del>E</del>H<del>L</del>E<del>U</del>]</td>
<td>1.7</td>
<td>2400</td>
<td>1.4 × 10⁻⁵</td>
</tr>
<tr>
<td>9</td>
<td>LBA1148 [pClo<del>E</del>B<del>E</del>L<del>E</del>U~]</td>
<td>3.5</td>
<td>0</td>
<td>&lt;3.5 × 10⁻⁵</td>
</tr>
<tr>
<td>10</td>
<td>LBA1148 [pClo<del>E</del>C<del>E</del>L<del>E</del>U~]</td>
<td>2.5</td>
<td>0</td>
<td>&lt;2.5 × 10⁻⁵</td>
</tr>
</tbody>
</table>
transformation with a derivative of pClo-GUS carrying a hygromycin-resistance cassette (J. Escudero, unpublished data).

Plasmid pClo-GUS could also be transferred to tobacco cells from strain LBA1010, which carries a wild-type T-DNA in its pTi plasmid. Interestingly, tumour formation in Nicotiana glauca plants inoculated with this strain was not inhibited by the presence of CloDF13 (pCloLEU in this case; data not shown). Hence, contrary to IncQ (RSF1010-derivative) plasmids and the IncW plasmid pSa, the CloDF13 plasmid seems not to interfere in translocation of the T-DNA or the virulence proteins such as VirE2.

To investigate which virulence genes are required for transfer of plasmid pClo-GUS to plant cells we tested several bacterial vir-mutants. As expected, mutations in virA, virB, or virG prevented transfer (see Table 4, lines 4, 5 and 6, respectively), whereas bacteria mutated in virD2 (Table 4, lines 7 and 8) showed a transfer efficiency similar to that of the wild-type strains (see also Fig. 2). Unexpectedly, however, some of the results obtained with other bacterial mutants in tobacco were different from those seen in yeast: neither the virD4 mutant nor the virE2 mutant could deliver pCloGUS into tobacco (Table 4, lines 10–12; Fig. 2G and H). Apparently, both VirD4 and VirE2 proteins are crucial for the establishment of CloDF13 DNA transformation of plant but not yeast cells. It is worthy to note here that the detection of CloDF13 transfer does not.

### Table 4. Transient GUS expression in tobacco plants inoculated with different Agrobacterium strains.

<table>
<thead>
<tr>
<th>Line</th>
<th>Bacterial strain&lt;sup&gt;a&lt;/sup&gt; with pClo-GUS</th>
<th>vir Mutation&lt;sup&gt;b&lt;/sup&gt;</th>
<th>GUS Activity&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LBA248</td>
<td>No vir genes</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>LBA1010</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>LBA1100</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>GV3101</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>LBA1142</td>
<td>virA</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>LBA1143</td>
<td>virB</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>LBA1145</td>
<td>virG</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>LBA1147</td>
<td>virD2</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>AT.virD2</td>
<td>ΔvirD2</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>LBA1148</td>
<td>virD4</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>LBA1149</td>
<td>virE2</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>AT.virE2</td>
<td>ΔvirE2</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> The bacterial strain LBA1010 carries a wild-type pTi plasmid with oncogenic T-DNA. Bacterial strains LBA1142-LBA1148 are all derivatives of strain LBA1100 (15). The bacterial strain AT.virD2 is a derivative of GV3101 (pPM6000), engineered with a precise deletion of virD2 (31).

<sup>b</sup> Corresponds either to the gene in which the Tn3-HoHoI transposon is inserted or to a whole gene deletion.

<sup>c</sup> GUS activity was tested by a very sensitive histochemical assay in SR1 tobacco plantlets. Scores with ‘–’ correspond to no detection of GUS activity in the plant tissue. Scores with ‘+’ correspond to high GUS activity in the plant tissue, similarly to the results obtained when GUS was assayed with a T-DNA-containing construct [e.g. pBGS (26)] harboured by the particular strain.

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require the genomic integration of the incoming plasmid, neither in yeast nor in plant cells.

Bacterial donors mutated in virD4 and virE2 can be complemented by co-infecting the tobacco cells with bacterial helpers

From the results presented above it can be concluded that mutations in virD4 or virE2 of the bacterial donor abolish pClo-GUS transfer to tobacco recipient cells. As such, this result could imply the inability of the pClo-GUS intermediate complex to be exported from the bacterial donor, specifically to the plant cell, in the absence of functional VirD4 or VirE2 proteins (‘hypothesis a’). Alternatively, export from the bacterial cell into the plant cell did occur but these virD4 and virE2 mutations prevented the plasmid complex from being properly established and expressed (‘hypothesis b’). To discriminate between these two hypotheses we performed extracellular complementation assays, i.e. we tested for the restoration of the transfer-deficient phenotype of the vir mutants by co-infecting the tobacco plantlets with helper (either vir wild type or vir mutated) strains. First we tested whether pClo-GUS transfer to tobacco from the virD4 and virE2 mutants could be restored by the presence of bacteria carrying a wild-type set of virulence genes. We co-inoculated the tobacco seedlings with mixtures of two bacterial strains, either LBA1148 (virD4*) or AT.virE2 (virE2*), both carrying the plasmid pClo-GUS, together with LBA1100 (wild-type vir) as helper. As shown in Fig. 3, these co-inoculations led to strong blue staining in the tobacco cells, similar to that observed when the plasmid pClo-GUS was transferred from the wild-type vir strains LBA1010 or LBA1100. We can thus conclude that pClo-GUS DNA can be transported from virD4 and virE2 mutants into tobacco (‘hypothesis a’ is incorrect). Apparently the pClo-GUS transferred DNA-complex cannot be properly established in tobacco (‘hypothesis b’).

Establishment of Agrobacterium T-DNA in plant cells (but not yeast cells) requires the independent delivery of a set of virulence proteins including VirE2, VirF (Vergunst et al., 2000) and VirE3 (Schrammeijer et al., 2003). These translocated proteins can be separately introduced into plant cells from a helper strain (‘extracellular complementation’). The VirD4 protein is essential for virulence protein translocation. As expected from a coupling factor, virD4 mutants cannot be complemented extracellularly for T-DNA transfer. A virD4-mutated bacteria (strain LBA1148) co-inoculated with a wild-type helper strain in Nicotiana

**Fig. 3.** GUS activity in SR1 tobacco leaves after extra-cellular complementation experiments. A. SR1 plantlets were inoculated with a mixture (1 : 1) of the wild-type strain LBA1100 together with the virE2-mutant LBA1149 (pClo-GUS) strain. B. Same as in A but with a mixture (1 : 1) of the wild-type strain LBA1100 together with the virD4-mutant LBA1148 [pClo-GUS] strain. C. Transgenic SR1 plantlets expressing virE2 were inoculated with the virE2-mutant LBA1149 (pClo-GUS) strain. D. Same as in C but inoculated with the virD4-mutant LBA1148 (pClo-GUS) strain.

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glauc\textit{a} leaf discs does not transfer T-DNA (GUS assay; J. Escudero, unpublished data).

Our results with the \textit{virE2} mutant thus suggest that for establishment of the pCloGUS DNA-complex in tobacco, the \textit{VirE2} protein needs to be translocated into the host cells. Thus we interpret the apparent extracellular complementation of the \textit{virD4} mutant for pCloGUS transfer to mean that the coupling protein encoded by CloDF13 can mediate transfer of the CloGUS DNA complex to tobacco, but cannot mediate the translocation of virulence proteins such as \textit{VirE2}, \textit{VirE3} and \textit{VirF}. Extracellular complementation by a helper strain provides these to establish pCloGUS transformation. In order to find out which of the translocated virulence proteins are essential for pCloGUS transformation, we performed extracellular complementation experiments of the \textit{virD4} mutant pCloGUS donor with helper strains mutated either in the \textit{virF} gene (LBA2561), or in the \textit{virE3} gene, or in both \textit{virF} and \textit{virE3} genes (data not shown). As transformation was as efficient as with the wild-type helper, the products encoded by \textit{virF} and \textit{virE3} seem to play only a minor role in the observed extracellular complementation. However, if the strain AT\textit{virE2} was used as helper, no GUS activity could be detected in the tobacco plantlets. These data showed that expression of \textit{virE2} in the helper strain was essential to bring about extracellular complementation of the pCloGUS transient expression. To find out whether \textit{VirE2} was the only essential factor delivered by the helper strain, we decided to inoculate SR1 transgenic tobacco plantlets that overexpressed the \textit{VirE2} protein with the pClo-GUS-containing \textit{virD4} or \textit{virE2} mutants as single donor strain. As expected, the inoculation of tobacco plantlets producing the \textit{VirE2} protein (two lines were tested, SR1/E2\#4 and SR1/E2\#13) with the \textit{virE2} mutant AT\textit{virE2} [pClo-GUS], resulted in a high proportion of plant cells with GUS activity (Fig. 3c). However, this was not the case when the \textit{virD4} mutant strain LBA1148 [pClo-GUS] was tested (Fig. 3D). These results indicated that the \textit{VirE2} protein is not the sole translocated virulence factor necessary for the establishment of CloGUS DNA in tobacco.

Discussion

We show here that a ~3.5 kb region from the plasmid CloDF13, designated \textit{oriT-mob}, encodes the functions required for \textit{trans}-kingdom DNA mobilization from \textit{Agrobacterium} to eukaryotic cells and that such mobilization has similar efficiency as that of T-DNA to yeast and tobacco cells. This is surprising as CloDF13 was originally isolated from \textit{E. cloacae} and is not a wide-host range plasmid. For transfer of CloDF13 neither the T-DNA-border repeats nor the \textit{VirD2} protein were necessary. The CloDF13 \textit{oriT} and the Mob relaxase can substitute for these functions. The \textit{VirD2} protein plays a major role not only in processing but also in piloting the T-complex from agrobacteria to the host-cell nucleus (reviewed in Rossi \textit{et al.}, 1998). The presence of a nuclear localization signal (NLS) in the C-terminal part of \textit{VirD2} is responsible for this. As transfer of CloDF13-derivative plasmids to both yeast and tobacco cells occurs efficiently, we must assume that the CloDF13 piloting relaxase also has such a NLS function. Besides \textit{VirD2}, the \textit{VirE2} protein is supposed to assist in the translocation of the T-complex through the nuclear envelope of plants by co-operatively covering the T-strand and at the same time protecting the T-DNA against nucleolytic attack. Our results suggest that \textit{VirE2} is likewise necessary for CloDF13 transfer to plants.

\textit{VirD4}-like proteins share common features. They are large proteins (~60 kDa) with NTP-binding motifs, associated with the bacterial cytoplasmic membrane and essential components for DNA transfer. Current models propose that \textit{VirD4}-like proteins act as ‘c coupling’ factors in the donor cell (de la Cruz and Lanka, 1998), bringing the nicked DNA of the relaxosome (e.g. processed T-complex) to the conjugal export machinery (e.g. VirB apparatus). Moreover, \textit{VirF} and \textit{VirE2} protein translocation has recently been shown to be dependent of a functional \textit{VirD4}/VirB system (Vergunst \textit{et al.}, 2000). Export of the T-complex requires both the VirB trans-membrane complex and the coupling factor \textit{VirD4}. However, CloDF13 brings dispensability for \textit{VirD4} as a novel feature in \textit{trans}-kingdom transfer. This resembles the finding that CloDF13 mobilization by the F plasmid does not require the TraD coupling factor. We observed that both the MobB and MobC proteins are essential for CloDF13 transfer to eukaryotic cells, although their precise role is not known. At least one of these proteins must be a coupling factor that can interact efficiently with the VirB channel to bring about plasmid DNA export and is likely recognized in the host cell for translocation across the nuclear envelope. However, neither MobB nor MobC of CloDF13 could complement the \textit{Agrobacterium virD4} mutant for T-DNA transfer or tumourigenesis (data not shown).

Our genetic data, reveal that CloDF13 can act as a novel interkingdom DNA-element and at the same time unravel a critical discriminatory role for the virulence factor \textit{VirD4} in \textit{Agrobacterium}-mediated transformation. Whereas \textit{VirD4} is unimportant for transformation of yeast, it appears essential for transformation of tobacco. What could be the distinct role of \textit{VirD4} in the genetic transformation of yeast and plant cells, respectively, by CloDF13? First, we investigated whether the general metabolic activity of the host cells was interfering with the observed results. One could imagine that dividing yeast cells in culture might present a different competence towards CloDF13 compared to the developmentally arrested tobacco cells in the plant tissue. Hence, tobacco proto-
plasts, a well-known transformable and proliferating plant-cell culture, were used instead of tobacco plantlets to assay for CloDF13 transfer. A similar requirement for VirD4 was found (data not shown), suggesting that the observed disparity between yeast and plant is species specific. One simple explanation might be that proper recognition of one of the CloDF13 Mob proteins does take place in yeast, thus substituting the Vir-protein counterpart, but not in plants. Nuclear targeting, nuclear entry or exposure to nuclease of the CloDF13 nucleoprotein complex might follow different requisites in yeast compared to plants. In fact, it is plausible that certain protein components (to date unknown, see below) to be transported from agrobacteria to the host cell via the VirD4/VirB apparatus are responsible for the observed differences. Thus, yeast would offer a ‘less stringent’ recipient cell than plant does, allowing transfer as well as expression of CloDF13 without the participation of such auxiliary bacterial proteins.

On the other hand, our results from the ‘extracellular’ complementation assays in tobacco cells evidenced that we could restore the absence of VirD4 and VirE2 in the donor cell by co-inoculation with (typically wild-type vir) helper bacteria. This was not due to bacterial conjugation because in control experiments we did not find transfer of CloDF13-derivative plasmids from Agrobacterium mutant donors to recipient helper strains (data not shown). These extracellular complementation assays excluded VirF but could not exclude VirE2 as the essential complementing factor. Nevertheless, when the virD4 mutated bacterial donor was inoculated into VirE2-expressing plants, transfer of CloDF13 could not be detected. Thus, VirE2 seems not to be the only player accountable for such a lack of transfer and other factors to be characterized, either vir or chromosome-encoded, are likely involved in Agrobacterium-mediated transformation of plants.

Because we did not observe a diminished virulence in agrobacteria containing CloDF13 we can conclude that the conjugal intermediate of plasmid CloDF13 was not inhibitory or competitive for T-DNA transfer. This is in sharp contrast to what has been observed with the IncQ plasmid RSF1010, conceived as a competitor/inhibitor of T-DNA transfer to plants (Binns et al., 1995). Contrary to CloDF13, RSF1010 does not contain any functional coupling protein. Therefore, taking together the transfer experiments in yeast and tobacco, we suggest that the proposed competition is not for VirB-export sites but rather for the coupling protein. Hence, RSF1010 transfer to eukaryotes likely relies on VirD4, just as vir-mediated mobilization of RSF1010 to other bacterial recipients requires VirD4 (Beijersbergen et al., 1992). In summary, our study leads us to suggest a simple model for mobilization of CloDF13 to higher organisms: upon initial processing, the nucleoprotein complex, probably via its Mob component and independently of VirD4, is recognized by the VirB export gate and transferred from agrobacteria without further requirements to yeast, where it is nuclear targeted. However, in plants some unknown bacterial protein is additionally needed in order to achieve either proper localization or DNA expression in the nucleus. Thus, the unusual characteristics of plasmid CloDF13 have helped to reveal the existence of a so far uncharacterized factor that might be responsible for the evolutionary specialization of agrobacteria to colonize plants.

Experimental procedures

Recombinant DNA techniques

Unless specified, standard protocols were followed for plasmid DNA isolation, cloning, restriction enzyme analysis and other standard molecular biology techniques (Sambrook et al., 1986). Total DNA from yeast was isolated using a method described elsewhere (Holm et al., 1986).

Plasmid constructions

In this study we adjusted to the more recent nomenclature for the mobilization genes of CloDF13 in accordance to the new sequencing data reported by Nufeze and de la Cruz (2001). However, it has to be noted that under this new sequence information the so-called mobA (Nufeze and de la Cruz, 2001) has not a clear-cut function in the conjugation process and what was originally baptized as mobA and mobB (Nijkamp et al., 1986) has apparently now been renamed mobB and mobC respectively.

We constructed pClo-LEU by insertion of: (i) a 4.6 kb BamHI–SalI region from plasmid CloDF13 (co-ordinates 1476–6624, anti-clock sense (Nijkamp et al., 1986)), containing the plasmid mobility region with the following elements: gene E (immunity protein), gene H (clavacin excretion protein), replication region, oriT, mobB and mobC; (ii) a 3.4 kb HindIII–SalI fragment from plasmid pBEJ16 (Hadfield et al., 1990) containing the 2 µm ori–STB region for replication and mitotic stability in S. cerevisiae plus leu2 as a yeast auxotrophic marker, into the IncP vector pRBJ (J. Escudero, unpublished data) which is a RK2 derivative plasmid containing a kanamycin gene for selection in bacteria. In Fig. 1 only the important elements for the trans-kingdom transfer described in this study are drawn. pClo-GUS (see also Fig. 1) was constructed similarly as pClo-LEU but in this case a 2.5-kb fragment containing the (CaMV 35S promoter-modified GUS–CaMV 35S terminator) gene cassette from plasmid pBG5 (Shen et al., 1993) was used as marker gene for specific expression in plants.

Bacterial and yeast strains

The Agrobacterium tumefaciens strains used in this work are listed in the Table 5. All bacterial strains contain the original C58 chromosomal background and either an octopine type pTiB6 plasmid with a wild-type vir-gene region or derivatives of it. The Escherichia coli strain used for cloning was DH5α.
Saccharomyces cerevisiae strain RSY12 (MATα leu2–3112 his3–1,15 ura3Δ::HIS3) was used as recipient cell in conjugation experiments with bacteria.

**Plasmid-DNA transfer assays**

Conjugation assays between agrobacteria and yeast were carried out as follows. The bacterial donor cells were grown for 2–3 days at 28°C on LC-agar (Hooykaas et al., 1979) medium plates in the presence of appropriate antibiotics (rifampicin, 10 mg l\(^{-1}\); kanamycin, 100 mg l\(^{-1}\); gentamycin, 80 mg l\(^{-1}\); carbenicillin, 75 mg l\(^{-1}\)). From fresh cultures, a single colony was inoculated into 10 ml of LC liquid medium with antibiotics. Growth was allowed overnight at 28°C with shaking to reach an OD\(_{600}\) between 1.0 and 1.5. Then bacteria were collected by centrifugation and washed with a 10 mM MgSO\(_4\) solution. Thereafter, bacteria were diluted to OD\(_{600}\) ~ 0.2 in two kinds of minimal liquid media: (i) MM (Hooykaas et al., 1979), which was adjusted to pH 7; (ii) IM containing the same composition as the MM, plus 0.5% (w/v) glycerol, 40 mM 2-(N-morpholino)ethanesulphonic acid (MES) and optional 0.2 mM AS, which was adjusted to pH 5.3. Bacteria in minimal liquid medium were further cultured for 8–10 h at 28°C with shaking, before being used for mating with the yeast cells. The yeast recipient cells were grown on YPD-agar (Sherman et al., 1983) medium plates and a single colony was cultured overnight at 30°C in YPD-liquid medium. Yeast cells were then diluted 20 times in fresh YPD liquid medium and subsequently cultured for 8–10 h at 30°C. Yeast cells were then collected by centrifugation and washed with either MM or with IM, then concentrated 10 times in the same medium before use. Subsequently, 50 µl of both the bacterial and yeast suspensions were gently mixed in an Eppendorf tube and finally placed on 0.45 µm cellulose nitrate filters. Bacteria-yeast conjugations were carried out either on MM-agar plates or on IM-agar plates, containing 5 mM glucose and the relevant aminoacids (leucine and uracil at 30 mg l\(^{-1}\) and histidine at 20 mg l\(^{-1}\)). After co-cultivation the filter with the cell mixture was immersed in 1 ml of PZ [physiological salt solution, 9 g l\(^{-1}\) (w/v) NaCl and shaken vigorously for 10–15 min. Afterwards, 100 µl aliquots of this conjugation mixture were plated out on MY-agar medium (Zonneveld, 1986) plates containing 0.2 mM cefotaxim, to counter select bacterial growth, and lacking leucine.

The number of Leu\(^+\) transformed RSY12 colonies obtained in this way, after incubation for one week at 30°C, was taken as an estimate of the efficiency of successful plasmid transfer from agrobacteria to yeast. The output number of bacteria (donor cells) and yeast (recipient cells) was accurately determined by plating out dilutions of the conjugation mixture in the PZ solution: for bacteria on LC-agar medium containing the relevant antibiotics and for yeast on MY-agar medium containing the full set of required aminoacids. Plasmid DNA was isolated from the Leu\(^+\) transformed yeast colonies and used to transform E. coli cells for a proper characterization by restriction analysis.

Plasmid pClo-GUS transfer assays to plants were carried out as follows. Agrobacteria were grown and treated as specified above for the conjugation assays with yeast, except that after washing with the 10 mM MgSO\(_4\) solution the bacterial suspension was adjusted to an OD\(_{600}\) ~ 1.0 in MS-liquid medium (Murashige and Skoog, 1962) before use. Tobacco seedlings (Nicotiana tabacum; cv. Petit Havana line SR1) 7 to 10 days old, from sterile in vitro germinated seeds in MS-agar medium, were used as plant-cell recipients. Routinely, 20 seedlings were immersed in 4 ml of bacterial suspension contained in plastic tubes and subjected to soft vacuum infiltration (~0.5 atm with occasional gentle shaking) during 15 min. Subsequently, the tobacco seedlings were quickly blotted on sterile paper and transferred to MS-agar medium plates containing 0.2 mM AS. Bacteria and plant co-cultivation was then allowed for 3 days in vitro at 23°C in a growth chamber with a 16 h light (2000 lux)/8 h dark regime. The tobacco plantlets were then washed in sterile distilled water and subjected to a GUS histochemical assay as described (Escudero et al., 1995). The number of tobacco cells expressing GUS was then taken as an estimation of the efficiency of pClo-GUS transfer from agrobacteria to the plant cell.

For every plasmid-transfer assay a number of experimental repeats was conducted (ranging between three and 10) and the data from a representative experiment are included in the respective Results section (see Tables 1–4).

**Tumour formation assays**

Agrobacteria were grown as described above on LC-agar medium with appropriate antibiotics. Bacterial cells were then...
resuspended in 10 mM MgSO₄ and adjusted to OD₆₀₀ – 1 in Eppendorf tubes before use. Two-months-old Nicotiana glauca plants were infected by puncturing first in the stem with a sterile toothpick and subsequently applying 20 µl of the bacterial suspension to be tested into the wound. Routinely three infections were performed per plant and every test was repeated in at least two plants. Furthermore, independent infection experiments were carried out with different batches of plants. After infection of the plants with bacteria, plant cell proliferation (the so-called tumour formation) was due to the oncogenic nature of the native T-DNA. Plants were scored for tumour formation after 6 weeks post infection.

**Extracellular complementation assays**

The particular mutation of the ‘donor’ bacterial strain was complemented by a ‘helper’ strain. Hence, two bacterial strains were used as inoculum: (i) the donor, carrying the particular vir-mutated pTi plasmid and pClo-GUS to be tested for transfer; (ii) the helper, without plasmid to be transferred but harbouring a set of virulence genes that could complement the specific mutation in the donor strain. Both donor and helper strains were inoculated at OD₆₀₀ – 1 using a 1:1 mixture. Besides, the virE2 as well as virD4 mutated strains were assayed for DNA transfer to transgenic SR1 tobacco plants expressing virE2.

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