Analysis of Vir protein translocation from *Agrobacterium tumefaciens* using *Saccharomyces cerevisiae* as a model: evidence for transport of a novel effector protein VirE3

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**ABSTRACT**

*Agrobacterium tumefaciens* causes crown gall disease on a variety of plants. During the infection process *Agrobacterium* transfers a nucleoprotein complex, the VirD2 T-complex, and at least two Vir proteins, VirE2 and VirF, into the plant cell via the VirB/VirD4 type IV secretion system. Recently, we found that T-DNA could also be transferred from *Agrobacterium* to *Saccharomyces cerevisiae*. Here, we describe a novel method to also detect trans-kingdom Vir protein transfer from *Agrobacterium* to yeast, using the Cre/lox system. Protein fusions between Cre and VirE2 or VirF were expressed in *Agrobacterium*. Transfer of the Cre-Vir fusion proteins from *Agrobacterium* to yeast was monitored by a selectable excision event resulting from site-specific recombination mediated by Cre on a lox-flanked transgene in yeast. The VirE2 and VirF proteins were transported to yeast via the *virB*-encoded transfer system in the presence of coupling factor VirD4, analogous to translocation into plant cells. The yeast system therefore provides a suitable and fast model system to study basic aspects of trans-kingdom protein transport from *Agrobacterium* into host cells. Using this method we showed that VirE2 and VirF protein transfer was inhibited by the presence of the Osa protein. Besides, we found evidence for a novel third effector protein, VirE3, which has a similar C-terminal signature to VirE2 and VirF.

**INTRODUCTION**

The plant pathogen *Agrobacterium tumefaciens* has a tumour-inducing (Ti) plasmid of which part, the transfer (T)-region, is transferred to plant cells during the infection process. As a result, the infected plant cells are triggered to divide, leading to the formation of crown gall tumours (reviewed in 1–8). The tumorous growth of the infected plant cells is caused by expression of the oncogenes located on the T-DNA. The vir region, also present on the Ti plasmid, encodes the Vir proteins, which mediate the processing of the T-region and the transfer of a single-stranded (ss) DNA copy of this region, the T-strand, into the recipient cells. One of the Vir proteins, the nicking enzyme VirD2, remains covalently attached to the 5′ end of the T-strand. VirD2 pilots the T-strand to the plant cell nucleus by virtue of its nuclear localisation signal (NLS). Recently, we showed that, in addition to the nucleoprotein complex, *A. tumefaciens* translocates the Vir proteins VirE2 and VirF directly into plant cells (9). Transport occurs via the VirB/VirD4 type IV secretion channel (7). The ssDNA-binding protein VirE2 and the F-box protein VirF play a role in the process of plant transformation. The VirE2 protein protects the T-strand and is involved in its transport into the nucleus (10). VirF can interact through its F-box with plant homologues of the yeast Skp1 protein (11). Skp1 and F-box proteins are subunits of a class of E3 ubiquitin ligases, called SCF complexes. These SCF complexes target specific proteins through interaction with F-box proteins for ubiquitin-mediated proteolysis (12,13). However, the target of VirF remains unknown so far. The *virF* operon contains a single gene, but the adjacent *virE* operon embraces three genes, namely *virE1, virE2* and *virE3*. The VirE1 protein plays an important role in the transport of VirE2 by acting as a chaperone of this protein and thus preventing at the same time premature binding to the T-strand and VirE2 aggregate formation (14,15). The function of the VirE3 protein has not been established, but *virF*vir*E3* double mutants are more strongly attenuated in virulence than *virF* mutants (B.Schrammeijer, P.Zuiderwijk and P.J.J.Hooykaas, unpublished results).

*Agrobacterium tumefaciens* can also transfer T-DNA to *Saccharomyces cerevisiae* (16) and filamentous fungi (17). The VirE2 and VirF proteins are not necessary for this process. The question was therefore raised whether *A. tumefaciens*...
nevertheless still translocates the Vir proteins VirE2 and VirF into yeast in a process that resembles translocation into plant cells. To study this, we made use of the site-specific recombination system Cre/lox of bacteriophage P1 (reviewed in 18), analogous to what was used to show direct Vir protein translocation from *Agrobacterium* into plants (9). Delivery of a bacterially expressed functional Cre recombinase fused to the VirE2 or VirF proteins into yeast cells was detected by selection for loss of a *lox* flanked (floxed) URA3 marker gene due to Cre recombination activity (Fig. 1). Our results indicate that *A. tumefaciens* indeed translocates VirE2 and VirF proteins directly into *S. cerevisiae* and that this translocation occurs via the VirB/VirD4 transport system as in plants. The yeast system thus offers a suitable and fast model system for the study of trans-kingdom protein translocation from *Agrobacterium*. We applied the system to show that the presence of the IncW plasmid pSa Osa protein, which inhibits tumour formation by *Agrobacterium* (19–21) blocks VirE2 and VirF protein transfer. Moreover we found evidence for the translocation of a third effector protein, the VirE3 protein, into yeast.

**MATERIALS AND METHODS**

**Construction of a S. cerevisiae strain containing a floxed URA3 gene**

The URA3 gene was cloned as a HindIII fragment from pJJ244 (22) into the filled-in EcoRI site of pIC-2lox (23) resulting in pSDM3011. The resulting floxed URA3 gene was subcloned as a HindIII fragment into pUC4x10 (24), which contains part of the *PDA1* locus (pSDM3012). Finally, the *PDA1-lox-URA3-lox-PDA1* cassette was cloned into binary vector pBin19 (25) resulting in pSDM3013. *Agrobacterium tumefaciens* strain LBA1126 (26) was electroporated (27) with *Agrobacterium* strain containing a functional Cre recombinase fused to a bacterially expressed functional Cre recombinase fused to a functional Cre enzyme from *Agrobacterium* into yeast is detected by Cre-mediated site-specific recombination on the *lox* sites resulting in loss of the URA3 gene allowing growth on medium containing FOA. Part of the mixture is used to determine the output of *Agrobacterium* and yeast cells (see Materials and Methods). Black triangles, *Agrobacterium* left and right border sequences.

**Construction of cre control and cre fusion plasmids**

**Cre control.** The *cre* recombinase gene was cloned as an *SphI/EcoRI* fragment from pUC19Cre (30) into pUC21 (31) (pSDM3120). The ATG start codon was removed by replacing the *BglII/NruI* fragment in pSDM3120 by a *BglII/NruI*-digested PCR fragment, amplified with primers cre 1 (5'-gtcgactATCGCCATCTTC-30 30 bp of *ura3* but lacking the stop codon of the *cre::virE2* fusion. The *virE2::cre* fusion. *VirE2* was fused translationally to the 5’ end of the *cre* gene in several steps. The *pVirE-virE1-virE2::cre* cassette from pSDM3122 was cloned into the *SmalI* sites of pRL662 (9) (pSDM3147 or cre control).

**cre::virE2 fusion.** To remove the STOP codon of the *cre* gene primers cre 6 (5'-acgcgtgatcATCGCAATCTTCCGAGCGCGC-30) and cre 7 (5'-cCATGATTTGTTA-CGGCGTAAGG-30) were used in a PCR amplification with promoter at the start codon position of *virE2* (pVirE-virE1-virE2). This cassette was cloned into the SmalI/XbaI sites of the broad host range, non-mobilisable plasmid pRL662 (9) (pSDM3147 or cre control).

Figure 1. Experimental strategy to detect Vir protein translocation from *Agrobacterium* into yeast. The yeast cells contain a *lox-URA3-lox* marker integrated at the *PDA1* locus. Upon co-cultivation with acetosyringone (AS)-induced *Agrobacterium* cells expressing Cre-Vir fusion protein (see Materials and Methods) for 6 days the mixture of cells is plated on medium supporting yeast growth and containing FOA. Delivery of a functional Cre enzyme from *Agrobacterium* into yeast is detected by Cre-mediated site-specific recombination on the *lox* sites resulting in loss of the URA3 gene allowing growth on medium containing FOA. Part of the mixture is used to determine the output of *Agrobacterium* and yeast cells (see Materials and Methods). Black triangles, *Agrobacterium* left and right border sequences.
pSDM3126 as template DNA. After digestion with ClaI (underlined) and SalI (underlined) the fragment was used to replace the corresponding fragment in pSDM3126 (pSDM3127 or creΔSTOP). An Xhol/SmalI fragment from pRAL3248 (32) was inserted into XhoI/EcoRV-digested pBluescriptIISK (34) followed by insertion of an XhoI/StuI linker (5′-tcgaGATCTTTCTGGCAATGAGAAATCGAG-3′) resulting in pBluevirE2ΔATG. VirE2ΔATG was then fused in frame to the 5′ end of cre as an XhoI/NorI fragment into the SalI/NorI sites of pSDM3127 (pSDM3128). Finally, a SalI/XhoI fragment was cloned into the Smal/XbaI sites of pRL662 resulting in pSDM3129 harbouring the pvirE-virE1-cre::virE2 cassette.

NLS::virF::cre fusion. A SacI/EcoRV 5′ virF flank from pTi1555, containing the ribosome binding site, was subcloned from pRAL7088 (35) into pBluescriptIISK (pSDM3183). The NLS from simian virus 40 (SV40) (34), containing an ATG start codon, was synthesised (Eurogentec, Belgium) with EcoRV and SalI sites, and cloned into pSDM3183 (pSDM3184). VirFΔATG 495–609 was cloned downstream the SV40 NLS in pSDM3184 (XhoI) from cloning a 175-bp fragment of the 3′ coding region was amplified with primers PT170 (5′-AATCCCTAACTTGGTCTTCAAC-3′) and PF583 (5′-cttgtacTACACCCGGCTTGATCGAG-3′) containing a BglII linker (5′-CTCAGGCCCCGCTTCAAC-3′) into pSDM3128 (pSDM3186). The 5′ virF::cre fusion was cloned into the XhoI site of pSDM3185 (pSDM3187 or NLS::virF::cre) and subsequently into SacI/PstI digested pUC28 (36). The final pvirF-NLS::virF::cre cassette was cloned into EcoRI digested pRL662 (pSDM3153).

NLS::cre::virF fusion. The 5′ virF flank–SV40NLS cassette from pSDM3184 was subcloned into pCI19H (SacI/SalI) (33), followed by a HindIII/SalI cloning into pCI19R and finally cloned as an XhoI/SalI fragment into the XhoI site of pSDM3121 (pSDM3188). Exchange of the ClaI/SalI 3′ cre fragment from pSDM3188 by the ClaI/SalI fragment from pSDM3127 resulted in a pvirF-NLS::creΔSTOP cassette (pSDM3179). VirFΔATG was cloned as a SalI/EagI fragment from pSDM3193 (11) into the SalI/NorI sites of pSDM3179 (pSDM3189). The pvirF-NLS::cre::virF cassette was finally inserted into HindIII/XbaI digested pRL662 (pSDM3154).

NLS::cre::virFΔ1–126 fusion. Deletion of the 126 5′ base pairs of the virF gene (Δ1–126) results in an N-terminal truncated VirFΔ2N protein lacking the F-box. The SalI/EagI fragment from pSM3194 (11) was cloned into the SalI/NorI sites of pSM3197, resulting in pSM3190 with a pvirF-NLS::cre::virFΔ1–126 cassette. This cassette was transferred into pRL662 as a HindIII/XbaI fragment (pSDM3155).

NLS::cre::virE3. The virE3 gene was cloned as an EcoRV/ SacI fragment from pRAL3248 (32) into pCI20R (33) resulting in pSDM3006. First, the 3′ coding region was subcloned in SalI/PstI digested pBluescriptIKS (pSDM3503). The ATG start codon was removed by PCR amplification with primers virE3-1 (5′-ACCGTCAGACATCTGGAGACTA-CAAAGAAAAG) and virE3-2B (5′-AGCCTTATTTCCGGCAAACC) with pSDM3006 as template DNA. SalI-digested PCR fragment (underlined) was cloned into pSDM3503 (pSDM3504). The final virE3ΔATG gene was ligated into SalI/XbaI digested pSDM3197, which resulted from cloning a HindIII/XbaI fragment from pSDM3179 in pRL662, and removal of the second SalI site in proximity to the virF promoter sequence. The final plasmid harbouring the pvirF-NLS::cre::virE3 cassette was named pSDM3507.

The in frame fusions of all plasmids as well as the accurate SV40NLS and 3′ virF fragment sequences were confirmed by sequence analysis.

Construction of the pOsa plasmid. The osa gene under control of the tac promoter was cloned from pUFR047::pTacoSA into pRL662 (pOsa or pSDM3180).

Agrobacterium tumefaciens strains

The stably maintained non-mobilisable plasmids pSDM3166 (virE2::cre), pSDM3129 (cre::virE2), pSDM3153 (NLS::virF::cre), pSDM3154 (NLS::cre::virF), pSDM3155 (NLS::cre::virFΔ1–126) and pSDM3507 (cre::virE3) were electroporated in Agrobacterium strain LBA1100, harbouring a disarmed octopine pTi plasmid named pAL1100 (37), according to den Dulk-Ras and Hooykaas (27). Furthermore, pSDM3129 and pSDM3155 were introduced in LBA2561, a LBA1100 derivative deleted for the virF gene (35), as well as in a series of LBA1100 derived vir mutants (37) having a Ths3oho1 transposon insertion in virA (LBA1142), virB4 (LBA1143), virB7 (LBA1144), virC2 (LBA1146), virD1 (LBA1150), virD2 (LBA1147), virD4 (LBA1148), virE2 (LBA1149) or virG (LBA1145). pNLS::cre::virFΔ1–126 was also introduced into LBA2565 (LBA1100::virE3). The strains LBA1147 and LBA1150 contained plasmid pSM3191 (or pD3,D4) to complement for polar mutation of downstream located virD3 and virD4. As control pSM3191 was introduced in LBA1100. pD3,D4 was made by cloning the virD3 and virD4 genes behind the virD promoter as a 4.4-kb BamHI fragment from pMP3 (38) into pSM3015. pSM3015 is a derivative of pBin19 (25) in which the T-region was replaced by the pIC19R/H polylinker sequence (33).

Agrobacterium strain LBA1100 was electroporated with the integrative cre control plasmid pSDM3126 or cre::virE2 fusion plasmid pSDM328, resulting in LBA1100::cre and LBA1100::cre::virE2, respectively. Selection for integration of the introduced plasmid via homologous recombination at the virE1 locus of pAL1100 was done on LC medium (10 g/l bacto-tryptone, 5 g/l bacto-yeast extract and 8 g/l NaCl, pH 7) containing carbenicillin (100 μg/ml) and integration was confirmed by Southern blot analysis. The same was performed for strain LBA2561 with plasmid pSDM3190, resulting in LBA2561::NLS::cre::virFΔ1–126 expressing the Cre::VirFΔ2N protein. Here, integration of the plasmid occurred at the 5′ virF flank of pAL1100::virF. pSDM3180 (pOsa) was introduced in LBA1100::cre::virE2 and LBA2561::NLS::cre::virFΔ1–126.
Protein transport experiments

Agrobacterium strains expressing the cre or cre-fusion gene were grown overnight at 30°C in 10 ml yeast-peptone-dextrose (YPD) medium (40). Cells were diluted 1:10 in fresh 100 ml YPD medium and grown for 5 h at 30°C. The output number of Saccharomyces cerevisiae LBY2 was determined after co-cultivation with and resuspended in 500 µl IM. Aliquots of 100 µl of the Agrobacterium and Saccharomyces cultures were mixed 1:1, the mixture was plated on 0.45 µm cellulose nitrate filters on PDA1 loci and resuspended in 2 ml of 40 mM 2-(N-morpholino) ethanesulfonic acid, pH 5.3, 10 mM glucose, 0.5% (w/v) glycerol and 200 µM acetoxyringone (AS). The cells were then grown for 5 h at 28°C.

Saccharomyces cerevisiae strain LBY2 was grown overnight at 30°C in 10 ml yeast-peptone-dextrose (YPD) medium (40). Cells were harvested and diluted at an A600 of 0.25 in 5 ml induction medium [IM: MM salts and 40 mM 2-(N-morpholino) ethanesulfonic acid, pH 5.3, 10 mM glucose, 0.5% (w/v) glycerol and 200 µM acetoxyringone (AS)]. The cells were then grown for 5 h at 28°C.

RESULTS

Translocation of the Cre::VirE2 and NLS::Cre::VirF fusion proteins from A.tumefaciens into S.cerevisiae

Agrobacterium tumefaciens can introduce T-DNA not only into plants but also into yeast and other fungi. In plants transfer of the Vir proteins VirE2 and VirF, which function in the plant cell in the process of tumour formation, accompanies T-DNA transfer. These transferred proteins are not essential for T-DNA transfer to yeast (16). We studied whether translocation of these proteins from A. tumefaciens into yeast occurs nevertheless as this might offer a fast and simple system for the study of this process of trans-kingdom protein translocation. To this end, we employed a similar strategy (see Fig. 1) as used to demonstrate protein translocation from Agrobacterium into plants (9). This strategy is based on the Cre-lox system from bacteriophage P1. Translational fusions of the cre gene with the 3' or 5' end of the virE2 and virF gene were placed under control of the virE and virF promoter, respectively. The SV40 NLS was added to the Cre-VirF fusion proteins to ensure nuclear targeting. The virE2 fusion genes were coordinately expressed with the virE1 gene. The cre gene transcriptionally fused to the virE1 gene and under control of the virE promoter was used as a control. To analyse protein transfer in the absence of T-DNA, the donor strains expressing the Cre fusion proteins contained a disarmed octopine Ti plasmid. The recipient yeast strain LBY2 carried a floxed URA3 gene integrated at the PDA1 locus on chromosome V. Delivery of a functional Cre enzyme into this yeast was monitored by Cre-mediated loss of the floxed URA3 gene due to site-specific recombination on the lox sites. Positive selection for loss of the URA3 gene was performed on medium containing FOA. Expression and recombination activity of the fusion proteins in A. tumefaciens was reported earlier (9). Results of a representative co-cultivation experiment are shown in Table 1. Co-cultivation with donor strains expressing the Cre recombinase alone, the VirE2::Cre or the NLS::VirF::Cre protein resulted in only a few Ura- colonies, which was similar to the number of Ura- colonies obtained after co-cultivation with an Agrobacterium strain lacking Cre altogether [Table 1; LBA1100 (none)]. However, when Cre was fused to the N-terminus of VirE2 (Cre::VirE2) or VirF (NLS::Cre::VirF) a large increase in the frequency of Ura- colonies per output recipient yeast (see Materials and Methods and legend to Table 1 for calculation of the frequency) was obtained (Table 1). These results show that delivery of Cre from A. tumefaciens into S. cerevisiae only occurs efficiently when Cre is fused to the N-terminus of VirE2 or VirF as is the case with transfer into plants.

Recently, we showed that VirF contains an F-box domain at its N-terminus by which it can bind to F-box interacting plant proteins (11). In co-cultivation experiments with plant cells, removal of the F-box from the NLS::Cre::VirF fusion protein (NLS::Cre::VirF(AA)) led to a 5-fold increase in the frequency of a Cre-mediated excision event, possibly due to an increased stability of the protein or the inability of the protein to bind with F-box interacting proteins in the cytoplasm (9). In transport experiments with yeast as a recipient, the F-box deleted version did not result in an increase in the number of colonies that had lost the URA3 gene (Table 1) compared with NLS::Cre::VirF. Furthermore, an NLS::Cre::VirF fusion protein in which the two most conserved amino acids of the F-box, leucine 26 and proline 27, were replaced by alanine [NLS::Cre::VirF(LP→AA)] did not result in an increased detection of excision (data not shown). Nevertheless, to be able to compare the transport studies in yeast and plants we used NLS::Cre::VirF(AA) for further transport studies to yeast.

Protein transport to yeast is dependent on the VirB/VirD4 secretion system

The results shown in the previous paragraph indicate that Agrobacterium translocates the VirE2 and VirF proteins into yeast. To analyse whether translocation from Agrobacterium into yeast has the same requirements as translocation into plants, the fusion proteins were expressed from a non-mobilisable broad host range plasmid in a set of vir mutants (donors) harbouring an octopine Ti plasmid lacking the T-region. Results of a representative co-cultivation experiment are given in Table 2A and B, respectively. Co-cultivation of yeast with donor strains carrying a mutation in virA or virG did not result in the generation of Ura- colonies at a frequency
The recipient yeast strain contains a floxed URA3 gene at chromosome V. Agrobacterium and yeast were co-cultivated for 6 days at 22°C and plated on medium containing 0.1% FOA.

Agrobacterium wild type strain LBA1100 (disarmed pTi) carried no (none) or a non-mobilisable plasmid expressing Cre alone (negative controls), VirE2-Cre, VirE2, NLS:VirE-Cre, NLS:Cre:VirF or NLS::Cre:VirFA2N, respectively.

After co-cultivation 100 μl of cells were plated (not diluted and 10^-2) on medium containing 0.1% FOA. The number of yeast Ura^+ colonies was determined after 4 days at 30°C. Full, too many colonies to count.

The output number of yeast and Agrobacterium cells was determined on LC and MY medium, respectively. For one Agrobacterium strain the output number was determined for a dilution of 10^-4.

The Cre-excision efficiency is determined by the number of yeast colonies on medium containing FOA per number of surviving yeast colonies (output yeast). The Cre-excision efficiency is determined by the number of yeast colonies on medium containing FOA per number of surviving yeast colonies (output yeast).

Table 1. Direct Vir protein translocation from Agrobacterium into yeast

<table>
<thead>
<tr>
<th>Agrobacterium strain</th>
<th>No. yeast colonies</th>
<th>Output yeast</th>
<th>Output Agro</th>
<th>Excision efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not diluted</td>
<td>10^-2 Dilution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1100 (none)</td>
<td>1</td>
<td>0</td>
<td>41</td>
<td>23</td>
</tr>
<tr>
<td>1100 (Cre)</td>
<td>3</td>
<td>0</td>
<td>95</td>
<td>14 x 10^-4</td>
</tr>
<tr>
<td>1100 (VirE2::Cre)</td>
<td>2</td>
<td>0</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>1100 (Cre::VirE2)</td>
<td>365</td>
<td>3</td>
<td>65</td>
<td>11</td>
</tr>
<tr>
<td>1100 (NLS::VirF::Cre)</td>
<td>4</td>
<td>0</td>
<td>86</td>
<td>25</td>
</tr>
<tr>
<td>1100 (NLS::Cre::VirF)</td>
<td>Full</td>
<td>151</td>
<td>124</td>
<td>19</td>
</tr>
<tr>
<td>1100 (NLS::Cre::VirFA2N)</td>
<td>Full</td>
<td>145</td>
<td>121</td>
<td>20</td>
</tr>
</tbody>
</table>

Agrobacterium wild type strain LBA1100 (disarmed pTi) and the wild type strain LBA1100 (disarmed pTi) carried no (none) or a non-mobilisable plasmid expressing Cre alone (negative controls), VirE2-Cre, Cre::VirE2, NLS::VirE-Cre, NLS::Cre:VirF or NLS::Cre::VirFA2N, respectively.

After co-cultivation 100 μl of cells were plated (not diluted and 10^-2) on medium containing 0.1% FOA. The number of yeast Ura^+ colonies was determined after 4 days at 30°C. Full, too many colonies to count.

The output number of yeast and Agrobacterium cells was determined on LC and MY medium, respectively. For three Agrobacterium strains the output number was determined for a dilution of 10^-4.

The Cre-excision efficiency is determined by the number of yeast colonies on medium containing FOA per number of surviving yeast colonies (output yeast). The Cre-excision efficiency is determined by the number of yeast colonies on medium containing FOA per number of surviving yeast colonies (output yeast).

Table 2. The effect of vir gene mutations

<table>
<thead>
<tr>
<th>Agrobacterium strain</th>
<th>No. yeast colonies</th>
<th>Output yeast</th>
<th>Output Agro</th>
<th>Excision efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not diluted</td>
<td>10^-2 Dilution</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| A. On Cre::VirE2 protein translocation from Agrobacterium into yeast
| 1100 (Cre)          | 6 | 0 | 25 | 5 | 2 x 10^-5 |
| 1100 (VirE2)        | Full | 11 | 24 | 73 | 5 x 10^-3 |
| virA (Cre::VirE2)   | 12 | 0 | 30 | 58 | 4 x 10^-5 |
| virB4 (Cre::VirE2)  | 26 | 0 | 37 | 7 x 10^-5 | 7 x 10^-5 |
| virB7 (Cre::VirE2)  | 5 | 0 | 17 | 66 | 3 x 10^-5 |
| virG (Cre::VirE2)   | 5 | 0 | 28 | 61 | 2 x 10^-5 |
| virC2 (Cre::VirE2)  | Full | 12 | 15 | 54 | 8 x 10^-3 |
| virD1 (Cre::VirE2)  | 5 | 0 | 16 | 98 | 3 x 10^-5 |
| 3VirD2 (Cre::VirE2) | 43 | 0 | 26 | 34 | 2 x 10^-4 |
| virD4 (Cre::VirE2)  | 5 | 0 | 18 | 16 | 3 x 10^-5 |
| virE2 (Cre::VirE2)  | Full | 23 | 19 | 39 | 1 x 10^-2 |
| virF (Cre::VirE2)   | Full | 8 | 25 | 72 | 3 x 10^-3 |
| virD1+pD3, D4 (Cre::VirE2) | Full | 13 | 46 | 72 | 3 x 10^-3 |
| 3VirD2+pD3, D4 (Cre::VirE2) | Full | 11 | 30 | 40 | 4 x 10^-3 |

B. On NLS::Cre:VirF42N protein translocation from Agrobacterium into yeast

| 1100 (Cre)          | 9 | 0 | 241 | 5 | 4 x 10^-6 |
| 1100 (NLS::Cre::VirF42N) | Full | 80 | 74 | 25 | 1 x 10^-2 |
| virA (NLS::Cre::VirF42N) | 1 | 0 | 43 | 15 | 2 x 10^-6 |
| virB4 (NLS::Cre::VirF42N) | 8 | 0 | 89 | 2 x 10^-5 | 9 x 10^-6 |
| virB7 (NLS::Cre::VirF42N) | 3 | 0 | 53 | 26 | 6 x 10^-6 |
| virG (NLS::Cre::VirF42N) | 27 | 0 | 387 | 74 | 7 x 10^-6 |
| virC2 (NLS::Cre::VirF42N) | Full | 51 | 74 | 12 | 7 x 10^-3 |
| virD1 (NLS::Cre::VirF42N) | 12 | 0 | 68 | 18 | 2 x 10^-5 |
| 3virD2 (NLS::Cre::VirF42N) | 30 | 0 | 55 | 29 | 5 x 10^-4 |
| virD4 (NLS::Cre::VirF42N) | 1 | 0 | 104 | 10 | 1 x 10^-6 |
| virE2 (NLS::Cre::VirF42N) | Full | 26 | 56 | 8 x 10^-5 | 5 x 10^-3 |
| virF (NLS::Cre::VirF42N) | Full | 102 | 76 | 18 | 1 x 10^-2 |
| virE3 (NLS::Cre::VirF42N) | Full | 80 | 81 | 30 | 1 x 10^-2 |
| 1100+pD3, D4 (NLS::Cre::VirF42N) | Full | 106 | 104 | 24 | 1 x 10^-2 |
| virD1+pD3, D4 (NLS::Cre::VirF42N) | Full | 131 | 115 | 19 | 1 x 10^-2 |
| 3virD2+pD3, D4 (NLS::Cre::VirF42N) | Full | 152 | 107 | 12 | 1 x 10^-2 |

Agrobacterium wild type strain LBA1100 (disarmed pTi) and the vir mutants carried a non-mobilisable plasmid expressing Cre alone (negative control), Cre::VirE2 (A) or NLS::Cre::VirF42N (B). All vir mutants, except for virF and virE2, which are deletion mutants, were obtained by Tn2 insertion. Plasmid pD3,D4 expresses the VirD3 and VirD4 proteins.

The recipient yeast strain contains a floxed URA3 gene at chromosome V. Agrobacterium and yeast were co-cultivated for 6 days at 22°C and plated on medium containing 0.1% FOA.

After co-cultivation 100 μl of cells were plated (not diluted and 10^-2) on medium containing 0.1% FOA. The number of yeast Ura^+ colonies was determined after 4 days at 30°C. Full, too many colonies to count.

The output number of yeast and Agrobacterium cells was determined on LC and MY medium, respectively. For three Agrobacterium strains the output number was determined for a dilution of 10^-4.

The Cre-excision efficiency is determined by the number of yeast colonies on medium containing FOA per number of surviving yeast colonies (output yeast). The Cre-excision efficiency is determined by the number of yeast colonies on medium containing FOA per number of surviving yeast colonies (output yeast).
virB7 or virD4 indicating that transport of VirE2 and VirF occurs via the VirB/VirD4 type IV secretion system (TFSS). Mutations in virD1 or virD2 led to a block and decrease, respectively, of protein transport, but as the transposon mutations in these genes were (partially) polar on the distal virD4 gene, this could be due to a defect in VirD4 expression. Therefore, we introduced a plasmid expressing VirD4 in these mutants and tested again for protein transport. This time the strains were proficient in Vir protein translocation into yeast, showing that neither virD1 nor virD2 are necessary for protein transfer. This was expected since VirD1 together with VirD2 is involved in efficient T-DNA processing and VirD2, by covalently binding the 5' end of the T-strand, also in transfer of the nucleoprotein complex from Agrobacterium into the host cell nucleus (8). Cre::Vir protein transport from Agrobacterium to yeast did take place from donor strains with mutations in the virC2 gene, the virE2 or virF genes. Apparently, the VirC2 and the wild type VirE2 and VirF proteins are not necessary for the transport of the fusion proteins. Furthermore, NLS::Cre::VirE2::VirF42N transport is independent of VirE3 (Table 2B) and VirE1 (data not shown). These results show that the VirB and VirD4 proteins that are required for VirE2 and VirF protein translocation into plants are equally important for Vir protein translocation into yeast. The yeast system thus offers a good model system to study protein translocation from Agrobacterium. We used it in the next two paragraphs to study transport of a putative third effector protein and to study the effect of tumourigenesis-inhibitor Osa on protein transport.

Identification of a third effector protein, VirE3

The translocated VirE2 and VirF proteins do not share an obvious transport domain. However, deletion analysis has shown that the transport signal is located in the C-terminal part of these proteins. A common Arg-Pro-Arg motif can be identified in this region and may form part of the transport signal (9). An Arg-Pro-Arg sequence is also present in the C-terminal region of the virulence protein VirE3. Although the function of this protein is still an enigma, it is worth noting that this protein has been conserved in all the different types of Ti and Ri plasmids. As the Arg-Pro-Arg motif indicated that VirE3 might be a transported effector protein of the Agrobacterium virulence system, we fused the N-terminus of VirE3 to NLS::Cre and assayed in the yeast system whether translocation of the fusion protein took place. The results of a representative experiment are summarised in Table 3. The data revealed that translocation into yeast cells did indeed occur with an efficiency intermediate between that of VirF and VirE2, thus identifying VirE3 as a third effector protein involved in the transformation of host cells by Agrobacterium. No transport of NLS::Cre::VirE3 was detected from a virD4 mutant, which is defective in type IV secretion (data not shown).

Osa inhibits Vir protein translocation from Agrobacterium into yeast

VirE2 or virF mutants (T-DNA donors) can be ‘extracellularly’ complemented for tumour formation on plants by coinfection with a helper strain lacking the T-region but containing the vir region including virE2 or virF, respectively (32,41–43). It has been found that the presence of the osa gene from IncW plasmid pSa in A.tumefaciens abolishes tumourigenicity on plants (44). Later, Lee et al. (45) showed that ‘extracellular’ complementation for tumour formation of a virE2 mutant on plants was inhibited by the presence of the osa gene in the helper strain but not by its presence in the T-DNA donor strain. These results indirectly indicate that Osa inhibits VirE2 transport rather than T-DNA transfer from Agrobacterium to plants. We have studied this possible inhibition of VirE2 and VirF transport by Osa in a direct assay. To this end, pOsa (pSDM3180) (containing the osa gene behind the tac promoter) was used in our protein transfer system to study the inhibitory effect of Osa on the translocation of Cre::VirE2 and NLS::Cre::VirF42N from Agrobacterium into yeast. Results of a representative cocultivation experiment are given in Table 4. Translocation of Cre::VirE2 or NLS::Cre::VirF42N from Agrobacterium into LBY2 resulted in a large increase of the frequency of Ura− colonies per output recipient compared with the control strain that expresses only Cre. In the presence of pOsa a complete inhibition of Cre::VirE2 and NLS::Cre::VirF42N translocation from Agrobacterium into yeast was observed, providing direct evidence for inhibition of Vir protein transport from Agrobacterium by Osa.

DISCUSSION

Here, we report a novel and fast method to detect A.tumefaciens Vir protein transport. We show that Agrobacterium not only transfers T-DNA to the yeast S.cerevisiae during co-cultivation (16) but also translocates the Vir proteins VirE2, VirE3 and VirF directly into yeast cells.

Virulence studies on plants have shown that an Agrobacterium strain mutated in the virE2 gene is avirulent on most plant species while a strain mutated in the virF gene is attenuated in virulence on certain host plants (43,46,47). The attenuated virulence of virF mutants is aggravated by a mutation in the virE3 gene (B.Schrammeijer, P.Zuiderwijk and P.J.J.Hooykaas, unpublished results). Both VirE2 and VirF play a role in the plant cell during tumourigenesis: plants expressing either VirE2 or VirF become hosts for virE2 and virF mutants, respectively (48,49). VirE2 attaches to the T-strand by cooperative binding thereby protecting the T-strand from degradation in the plant cell (50). Such coating is important for efficient entrance of the T-strand into the plant nucleus (8,10). VirF is an F-box protein that interacts with homologues of the yeast Skp1 protein in plants (11). F-box proteins and Skp1 form a part of SCF complexes, which are responsible for the ubiquitin-mediated proteolysis of specific plant proteins. VirF as part of such an SCF complex in plants, may be involved in the targeted proteolysis of specific host proteins during tumourigenesis by A.tumefaciens (11).

Recently, it was shown in our group that T-DNA transfer from virE2 or virF mutants to S.cerevisiae was not or only slightly affected compared with the wild type strain (16) (H. van Attikum, personal communication). Here, we show that although VirE2 and VirF are not essential for T-DNA transfer from Agrobacterium to yeast these Vir proteins are still translocated into yeast. Translocation of VirE2 and VirF fusion proteins from Agrobacterium into yeast occurs via the
Table 3. Detection of VirE3 protein translocation from Agrobacterium into yeast

<table>
<thead>
<tr>
<th>Agrobacterium strainb</th>
<th>No. yeast coloniesc</th>
<th>Output yeastd</th>
<th>Output Agroe</th>
<th>Excision efficiencyf</th>
</tr>
</thead>
<tbody>
<tr>
<td>1100 (Cre)</td>
<td>6</td>
<td>201</td>
<td>94</td>
<td>3 x 10^-6</td>
</tr>
<tr>
<td>1100 (NLS::Cre::VirF42N)</td>
<td>115</td>
<td>326</td>
<td>76</td>
<td>3 x 10^-3</td>
</tr>
<tr>
<td>1100 (Cre::VirE2)</td>
<td>250</td>
<td>31</td>
<td>8 x 10^-5</td>
<td>1 x 10^-4</td>
</tr>
<tr>
<td>1100 (NLS::Cre::VirE3)</td>
<td>275</td>
<td>85</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aThe recipient yeast strain contains a floxed URA3 gene at chromosome V. Agrobacterium and yeast were co-cultivated for 6 days at 22°C and plated on medium containing 0.1% FOA.
bAgrobacterium strain LBA1100 (wild type, disarmed pTi) carried a non-mobilisable plasmid expressing Cre alone (negative control), NLS::Cre::VirF42N, Cre::VirE2 or NLS::Cre::VirE3, respectively.
cAfter co-cultivation 100 µl of cells were plated (not diluted and 10^-2) on medium containing 0.1% FOA. The number of yeast Ura^- colonies was determined after 4 days at 30°C. Full, too many colonies to count.
dThe output number of yeast and Agrobacterium cells was determined on LC and MY medium, respectively.
eThe Cre-excision efficiency is determined by the number of yeast colonies on medium containing FOA per number of surviving yeast colonies (output yeast).

Table 4. The inhibitory effect of the Osa protein on Cre::Vir protein translocation from Agrobacterium into yeast

<table>
<thead>
<tr>
<th>Agrobacterium strainb</th>
<th>No. yeast coloniesc</th>
<th>Output yeastd</th>
<th>Output Agroe</th>
<th>Excision efficiencyf</th>
</tr>
</thead>
<tbody>
<tr>
<td>1100 (none)</td>
<td>1</td>
<td>41</td>
<td>23</td>
<td>2 x 10^-6</td>
</tr>
<tr>
<td>1100::cre</td>
<td>1</td>
<td>39</td>
<td>41</td>
<td>2 x 10^-6</td>
</tr>
<tr>
<td>1100::cre::virE2</td>
<td>416</td>
<td>52</td>
<td>29</td>
<td>8 x 10^-4</td>
</tr>
<tr>
<td>1100::cre::virE2 + pOsa</td>
<td>0</td>
<td>61</td>
<td>18</td>
<td>&lt;2 x 10^-5</td>
</tr>
<tr>
<td>2561::NLS::cre::VirD1-126</td>
<td>Full</td>
<td>36</td>
<td>46</td>
<td>1 x 10^-5</td>
</tr>
<tr>
<td>2561::NLS::cre::virD1-126 +pOsa</td>
<td>7</td>
<td>71</td>
<td>17</td>
<td>1 x 10^-5</td>
</tr>
</tbody>
</table>

aThe recipient yeast strain contains a floxed URA3 gene at chromosome V. Agrobacterium and yeast were co-cultivated for 6 days at 22°C and plated on medium containing 0.1% FOA.
bAgrobacterium wild type strain LBA1100 (disarmed pTi) expressing no Cre (none), LBA1100::cre expressing Cre alone (negative controls), and LBA1100::cre::virE2 expressing Cre::VirE2 or Cre::VirE2 + Osa, respectively. LBA2561::NLS::cre::VirD1-126 expressing NLS::Cre::VirF42N or NLS::Cre::VirF42N + Osa, respectively. The genes were integrated by a single cross over in the Ti-plasmid.
cAfter co-cultivation 100 µl of cells were plated (not diluted and 10^-2) on medium containing 0.1% FOA. The number of yeast Ura^- colonies was determined after 4 days at 30°C. Full, too many colonies to count.
dThe output number of yeast and Agrobacterium cells was determined on LC and MY medium, respectively.
eThe Cre-excision efficiency is determined by the number of yeast colonies on medium containing FOA per number of surviving yeast colonies (output yeast).

VirB secretion system in the presence of coupling factor VirD4 as was shown for plants (9). This indicates that both Vir proteins are actively translocated through the same pore/pilus like structure through which the T-strand is transferred into the yeast cell (16). In addition, we found now that VirE3 is also transported through this VirB/D4 channel.

The VirB/VirD4 transport system of A.tumefaciens is the prototype of the TFSS (reviewed in 7). Other members of this family of TFSSs are the conjugative DNA transport systems encoded by the (broad host range) plasmids IncW, IncN and IncP. In several animal pathogens, like Helicobacter pylori, Bordetella pertussis, Legionella pneumophila, Rickettsia prowazekii and Brucella suis, similar secretion systems are present that may not be involved in DNA transfer, but in protein export. Evidence for protein secretion has been found for the Bordetella and Helicobacter systems (reviewed in 51). It is entirely possible that the TFSSs involved in DNA translocation have evolved from a protein transport system. In this scenario the relaxase bound to the 5'-end of the DNA acts as a pilot protein that is recognised by the TFSS.

Translocation of VirE2 is independent of VirF and translocation of VirF is independent of VirE2 and VirE3. The VirE3 protein is encoded by a gene, which was identified downstream of the virE2 gene on Ti plasmids (52–54). Mutations in virE3 aggravate the attenuated virulence of virF mutants. We now show that translocation of VirF into yeast is not affected by the absence of VirE3 from the bacterium, and that VirE3 itself is a translocated effector protein. Further study is necessary to reveal the role of VirE3 during tumourigenesia on plants.

For delivery of Cre into yeast cells mediated by VirE2 or VirF transport signals, the Cre protein must be fused to the N-terminus of either Vir protein, as was the case for delivery of Cre into plant cells (9). Interestingly, the 37 C-terminally located amino acids of VirF were sufficient to introduce Cre into yeast (data not shown), as in plants (9). These results show that the same transport signals located in the C-terminus of VirE2 and VirF are important to mediate translocation into plant and yeast cells. In agreement with a C-terminal transport signal, VirE3 was able to transport Cre into yeast as an N-terminal fusion. The common Arg-Pro-Arg motif that is present in the C-termini of VirE2, VirE3 and VirF may form part of the transport domain. Currently, we are analysing the C-terminal signal sequences in more detail.

To ensure nuclear targeting of the Cre::VirF fusion constructs the SV40 NLS sequence was fused translationally to the N-terminus. Guralnick et al. (55) suggested that the NLS sequences present in VirE2 are unable to mediate entrance into the nucleus of Drosophila embryos or Xenopus oocytes and that they are rather plant specific. Recently, Rhee et al. (56) showed in a genetic assay the inability of the VirE2 NLS to function in nuclear import in yeast cells. Here, we show that
the Cre::VirE2 fusion protein is able to enter the yeast cell nucleus. Therefore, nuclear targeting is most probably mediated by the cryptic NLS sequence present in Cre (57).

Removal of the N-terminus from VirF, including the F-box (42 amino acids), resulted in a 5-fold higher efficiency of Cre-mediated deletion in Arabidopsis (9). The same high efficiency was obtained in Arabidopsis for the NLS::Cre::VirF(LP→AA) fusion protein (data not shown). Deletion or mutation of the F-box from VirF may stabilise the VirF fusion protein in plants by preventing VirF from binding to F-box interacting proteins (Skp1 orthologs) in the plant cytoplasm (11), ultimately leading to an increase in the numbers of NLS::Cre::VirFΔ42N or NLS::Cre::VirF(LP→AA) molecules that reach the plant cell nucleus (9). However, the absence or mutation of this F-box from VirF did not lead to the detection of enhanced Cre activity in yeast. This difference might be the result of distinct binding affinities in yeast and plants between the full length VirF protein and the Skp1 orthologs, making the full length VirF protein more stable in yeast. Another explanation might be that a different factor, such as the number of yeast cells competent for transformation, is limited for translocation to yeast, thus concealing the effect of deletion of the F-box.

Previously, it was suggested that oncogenic suppression by Osa involves inhibition of VirE2 transport rather than T-DNA transfer from Agrobacterium to plants (45). We have confirmed this in our direct protein transport assay. Moreover, we have shown that inhibition of protein transfer is not restricted to VirE2, but involved at least VirF also. The molecular mechanism by which Osa inhibits protein transport is unknown. In a previous article it was proposed that Osa may do this by interacting with VirE1, which is needed for VirE2 transport from the bacterium (45). However, as transport of VirF, which does not need VirE1 (data not shown), is inhibited by Osa we also think it is unlikely that Osa’s inhibitory effects are a result of its interaction with VirE1. Recently, we have done experiments to see whether Osa may compete with VirE2 and VirF export by being a favoured translocation protein itself. However, preliminary results indicate that Osa is not transported by the VirB/VirD4 secretion channel (data not shown). Therefore, at the moment we favour the idea that Osa, being a membrane protein, either inhibits the function of one of the proteins of the VirB/VirD4 type IV secretion channel or binds to the transported Vir proteins (VirE2, VirF) and thereby prevents these from being exported. Further research has to clarify the mechanism by which Osa inhibits Vir protein transport from Agrobacterium into the host cell.

Our results indicate that the Agrobacterium system, which has so far been used as a tool to introduce genes of interest into plants, yeast and other fungi, also offers potential for fast and reliable alternative for translocation studies of virulence effectors from bacteria carrying a TSS.

ACKNOWLEDGEMENTS

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REFERENCES

15. Zhao,Z., Sagulenko,E., Ding,Z. and Christie,P.J. (2001) Activities of virE1 and the VirE1 secretion chaperone in export of the multifunctional (e.g. lox-kanMX-lox) for recombinases (e.g. Cre recombinase). The selection marker is now removed by introducing a plasmid expressing the Cre protein in the yeast cell. Furthermore, this plasmid has to be removed again from the yeast strain. The method of protein transport (of a functional recombinase) described in this paper avoids the need to introduce and subsequently remove a Cre expressing plasmid. Therefore, with this Cre-Vir-lox system we provide a fast method to mediate site-specific recombination events in yeast. Besides, the trans-kingdom protein transfer to yeast offers a fast and reliable alternative for translocation studies of virulence effectors from bacteria carrying a TSS.