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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 *virFvirE3* 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*

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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 (f*lox*ed) *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.

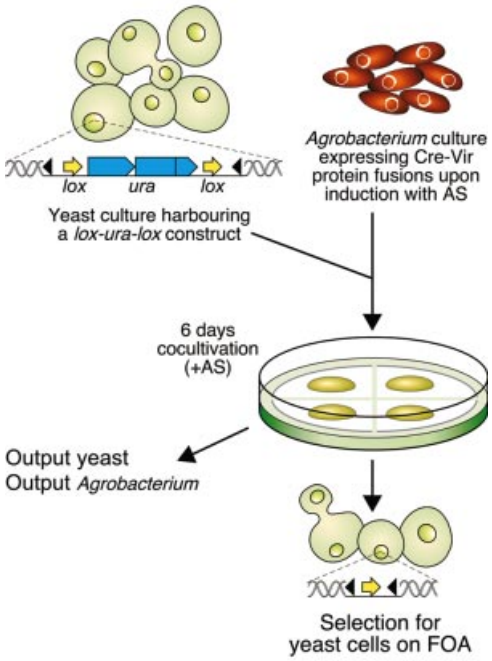

Figure 1. Experimental strategy to detect Vir protein translocation from *Agrobacterium* into yeast. The yeast cells contain a *lox-URA3-lox* marker integrated at the *PDA* locus. Upon co-cultivation with acetosyringone (AS)-induced *Agrobacterium* cells expressing Cre-Vir fusion protein (see Materials 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.

MATERIALS AND METHODS

Construction of a *S.cerevisiae* strain containing a f*lox*ed URA3 gene

The *URA3* gene was cloned as a *Hin*dIII fragment from pJJ244 (22) into the filled-in *Eco*RI site of pIC-2lox (23) resulting in pSDM3011. The resulting f*lox*ed *URA3* gene was subcloned as a *Hin*dIII fragment into pUC4α10 (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 pSDM3013 and used in a co-cultivation experiment with *S.cerevisiae* strain RSY12 (*MAT***a** *leu2-3, 112 his3-11, 15 ura3Δ::HIS3*) (28). Transformants, prototrophic for uracil, were selected on minimal yeast (MY) medium (29) containing 200 μM cefotaxim (Duchefa, B.V.), leucine (30 mg/l) and histidine (20 mg/l) but lacking uracil. Transformants were further characterised with polymerase chain reaction (PCR) and Southern blot analysis. Strain LBY2, in which the *loxURA3lox* gene had integrated at the *PDA1* locus on chromosome V by homologous recombination (gene replacement), was selected and used in all co-cultivation experiments.

Construction of cre control and cre fusion plasmids

Cre control. The *cre* recombinase gene was cloned as an *Sph*I/*Eco*RI fragment from pUC19Cre (30) into pUC21 (31) (pSDM3120). The ATG start codon was removed by replacing the *Bgl*II/*Nru*I fragment in pSDM3120 by a *Bgl*II/*Nru*I-digested PCR fragment, amplified with primers cre 1 (5′-ggcagatctgTCCAATTTACTG-3′) and cre 2 (5′-GATAA-TCGCGAACATCTTCAGG-3′) (restriction sites underlined) using pSDM3120 as template (pSDM3121). A 2.2-kb *Sal*I fragment from pRAL3248 (32), containing *virE* promoter, *virE1* and *virE2*(Δ*30 3′* bp), was cloned into the *Xho*I site of pSDM3121 (pSDM3122). Digestion with *Bgl*II and *Bst*YI (partial) followed by ligation of the vector (pSDM3126) resulted in transcriptional control of *cre* from the *virE* promoter at the start codon position of *virE2* (*pvirE-virE1-cre*). This cassette was cloned into the *Sma*I/*Xba*I sites of the broad host range, non-mobilisable plasmid pRL662 (9) (pSDM3147 or cre control).

virE2::cre fusion. VirE2 was fused translationally to the 5′ end of the *cre* gene in several steps. The *pvirE-virE1-virE2*(Δ*30 3′* bp)*::cre* cassette from pSDM3122 was cloned into the *Sma*I/*Xba*I sites of pRL662 (pSDM3148). The 3′ end of the *virE2* gene was restored as follows. The *Bgl*II/*Nru*I fragment of pSDM3122 (*cre1-205* Δ*ATG*) was cloned into pIC19R (33) and named pSDM3151. A 34-bp *Sal*I/*Bgl*II linker (5′-TCGACCGCGTAGCCAAAGCGTCAACAGCTTTCGA-3′) representing the 3′ 30 bp of *virE2* but lacking the stop codon was cloned into pSDM3151 (pSDM3152). The *Sal*I fragment of pRAL3248 [*pvirE-virE1-virE2*(Δ*30 3′* bp)] was cloned into pSDM3152 resulting in a translational fusion of full length *virE2* with *cre* (pSDM3157). The *Nru*I fragment in pSDM3148 was finally replaced by the *Nru*I fragment of pSDM3157 resulting in plasmid pSDM3166 carrying the *pvirE-virE1-virE2::cre* cassette.

cre::virE2 fusion. To remove the STOP codon of the *cre* gene primers cre 6 (5′-acgcgtcgactATCGCCATCTTC-CAGCAGGCGC-3′) and cre 7 (5′-cCATCGATTGATTA-CGGCGCTAAGG-3′) were used in a PCR amplification with

pSDM3126 as template DNA. After digestion with *Cla*I (underlined) and *Sal*I (underlined) the fragment was used to replace the corresponding fragment in pSDM3126 (pSDM3127 or creΔSTOP). An *Xho*I/*Sma*I fragment from pRAL3248 (32) was inserted into *Xho*I/*Eco*RV-digested pBluescriptIISK⁻ (34) followed by insertion of an *Xho*I/*Stu*I linker (5′-tcgaGATCTTTCTGGCAATGAGAAATCAGG-3′) resulting in *pBluevirE2ΔATG*. *VirE2ΔATG* was then fused in frame to the 3′ end of *cre* as an *Xho*I/*Not*I fragment into the *Sal*I/*Not*I sites of pSDM3127 (pSDM3128). Finally, a *Stu*I/*Xba*I fragment was cloned into the *Sma*I/*Xba*I sites of pRL662 resulting in pSDM3129 harbouring the *pvirE-virE1-cre::virE2* cassette.

NLS::virF::cre fusion. A *Sac*I/*Eco*RV 5′ *virF* flank from pTi15955, containing the ribosome binding site, was subcloned from pRAL7088 (35) into pBluescriptIISK⁻ (pSDM3183). The NLS from simian virus 40 (SV40), containing an ATG start codon, was synthesised (Eurogentec, Belgium) with *Eco*RV and *Sal*I sites, and cloned into pSDM3183 (pSDM3184). *VirFΔATGΔ495–609* was cloned downstream the SV40 NLS in pSDM3184 (*Xho*I) as a *Sal*I/*Xho*I fragment from pSDM3193 (11) and named pSDM3185. To remove the STOP codon in *virF* a 175-bp fragment of the 3′ coding region was amplified with primers PF170 (5′-ATCCCTAACTTGGTCTTCAAC-3′) and PF583 (5′-cttagatcTAGACCGCGCGTTGATCGAGG-3′) containing a *Bgl*II site at the 5′ end (underlined) using pRAL7088 (35) as template DNA. After subcloning the PCR fragment into the pGEM T-vector (Promega) *VirFΔSTOP* was cloned into *Xho*I/*Bgl*II-digested pSDM3121-L, which resulted after insertion of a *Stu*I/*Bgl*II linker (5′-CCTCGAGCCCGGGATA-3′) into pSDM3121 (pSDM3186). The 3′ *virF::cre* fusion was cloned into the *Xho*I site of pSDM3185 (pSDM3187 or *NLS::virF::cre*) and subsequently into *Sac*I/*Pst*I digested pUC28 (36). The final *pvirF-NLS::virF::cre* cassette was cloned into *Eco*RI digested pRL662 (pSDM3153).

NLS::cre::virF fusion. The 5′ *virF* flank–*SV40NLS* cassette from pSDM3184 was subcloned into pIC19H (*Sac*I/*Sal*I) (33), followed by a *Hin*dIII/*Sal*I cloning into pIC19R and finally cloned as an *Xho*I/*Sal*I fragment into the *Xho*I site of pSDM3121 (pSDM3188). Exchange of the *Cla*I/*Sal*I 3′ *cre* fragment from pSDM3188 by the *Cla*I/*Sal*I fragment from pSDM3127 resulted in a *pvirF-NLS::creΔSTOP* cassette (pSDM3179). *VirFΔATG* was cloned as a *Sal*I/*Eag*I fragment from pSDM3193 (11) into the *Sal*I/*Not*I sites of pSDM3179 (pSDM3189). The *pvirF-NLS::cre::virF* cassette was finally inserted into *Hin*dIII/*Xba*I 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 VirFΔ42N protein lacking the F-box. The *Sal*I/*Eag*I fragment from pSDM3194 (11) was cloned into the *Sal*I/*Not*I sites of pSDM3179, resulting in pSDM3190 with a *pvirF-NLS::cre::virFΔ1–126* cassette. This cassette was transferred into pRL662 as a *Hin*dIII/*Xba*I fragment (pSDM3155).

NLS::cre::virE3. The *virE3* gene was cloned as an *Eco*RV/*Sac*I fragment from pRAL3248 (32) into pIC20R (33) resulting in pSDM3006. First, the 3′ coding region was subcloned in *Sal*I/*Pst*I digested pBluescriptIIKS (pSDM3503). The ATG start codon was removed by PCR amplification with primers virE3-1 (5′-ACGCgtcgacagatctGCGTGAGCACTA-CGAAGAAAAG) and virE3-2B (5′-AGCCTATTTCGC-CACGAAACCC) with pSDM3006 as template DNA. *Sal*I-digested PCR fragment (underlined) was cloned into pSDM3503 (pSDM3504). The final *virE3ΔATG* gene was ligated into *Sal*I/*Xba*I digested pSDM3197, which resulted from cloning a *Hin*dIII/*Xba*I fragment from pSDM3179 in pRL662, and removal of the second *Sal*I 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 Tn3*hoho1* transposon insertion in *virA* (LBA1142), *virB4* (LBA1143), *virB7* (LBA1144), *virC2* (LBA1146), *virD1* (LBA1150), *virD2* (LBA1147), *virD4* (LBA1148), *virE2* (LBA1149) or *virG* (LBA1145). p*NLS::cre::virFΔ1–126* was also introduced into LBA2565 (LBA1100Δ*virE3*). The strains LBA1147 and LBA1150 contained plasmid pSDM3191 (or pD3,D4) to complement for polar mutation of downstream located *virD3* and *virD4*. As control pSDM3191 was introduced in LBA1100. pD3,D4 was made by cloning the *virD3* and *virD4* genes behind the *virD* promoter as a 4.4-kb *Bam*HI fragment from pMP3 (38) into pSDM3015. pSDM3015 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 pSDM3128, 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::VirFΔ42N protein. Here, integration of the plasmid occurred at the 5′ *virF* flank of pAL1100Δ*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 29°C in 5 ml minimal medium (MM) (39) supplemented with spectinomycin (250 µg/ml) and, in case a pRL662-based plasmid was present, also with gentamycin (40 µg/ml). 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 acetosyringone (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 1:10 diluted in fresh 100 ml YPD medium and grown for 5 h at 30°C. The cells were subsequently washed with and resuspended in 500 µl IM. Aliquots of 100 µl of the *Agrobacterium* and *Saccharomyces* cultures were mixed 1:1, the mixture was placed on 0.45 µm cellulose nitrate filters on IM containing 5 mM glucose and the amino acids leucine and uracil at a concentration of 30 mg/l. To prevent loss of pRL662 gentamycin was added to the medium. After 6 days of co-cultivation at 22°C, the mixture was resuspended in 2 ml NaCl solution (9 g/l) and 100 µl aliquots (undiluted and a 10^{-2} dilution) were plated out on solid MY medium supplemented with leucine, uracil, 200 µM cefotaxim and 0.1% fluoro-orotic acid (FOA). The number of colonies that had lost the *URA3* gene as indicated by growth on FOA were calculated after 4 days growth at 30°C. The output number of *Agrobacterium* cells (n.b. of viable cells after co-cultivation) was determined on solid LC medium supplemented with spectinomycin or gentamycin by plating a 10^{-5} and a 10^{-6} dilution of the co-cultivation mix. The output number of *Saccharomyces* cells was determined on solid MY medium with cefotaxim and the amino acids leucine and uracil by plating a 10^{-4} and a 10^{-5} dilution of the co-cultivation mix. The efficiency of excision of the *URA3* gene is indicated as the number of yeast colonies on FOA divided by the number of surviving yeast colonies (output yeast).

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 f*lox*ed *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 f*lox*ed *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::VirFΔ42N) 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::VirFΔ42N 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

Table 1. Direct Vir protein translocation from *Agrobacterium* into yeast[a]

Agrobacterium strain[b]	No. yeast colonies[c] Not diluted	10^{-2} Dilution	Output yeast[d] 10^{-4}	Output Agro[e] 10^{-6}	Excision efficiency[f]
1100 (none)	1	0	41	23	2×10^{-6}
1100 (Cre)	3	0	95	14 (10^{-4})	3×10^{-6}
1100 (VirE2::Cre)	2	0	78	23	3×10^{-6}
1100 (Cre::VirE2)	365	3	65	11	6×10^{-4}
1100 (NLS::VirF::Cre)	4	0	86	25	5×10^{-6}
1100 (NLS::Cre::VirF)	Full	151	124	19	1×10^{-2}
1100 (NLS::Cre::VirFΔ42N)	Full	145	121	20	1×10^{-2}

[a]The recipient yeast strain contains a *flox*ed *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.
[b]*Agrobacterium* wild type strain LBA1100 (disarmed pTi) carried no (none) or a non-mobilisable plasmid expressing Cre alone (negative controls), VirE2::Cre, Cre::VirE2, NLS::VirF::Cre, NLS::Cre::VirF or NLS::Cre::VirFΔ42N, respectively.
[c]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.
[d,e]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}.
[f]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

Agrobacterium strain[a]	No. yeast colonies[c] Not diluted	10^{-2} Dilution	Output yeast[d] 10^{-4}	Output Agro[e] 10^{-6}	Excision efficiency[f]
A. On Cre::VirE2 protein translocation from *Agrobacterium* into yeast[b]					
1100 (Cre)	6	0	25	5	2×10^{-5}
1100 (Cre::VirE2)	Full	11	24	73	5×10^{-3}
virA (Cre::VirE2)	12	0	30	58	4×10^{-5}
virB4 (Cre::VirE2)	26	0	37	7 (10^{-5})	7×10^{-5}
virB7 (Cre::VirE2)	5	0	17	66	3×10^{-5}
virG (Cre::VirE2)	5	0	28	61	2×10^{-5}
virC2 (Cre::VirE2)	Full	12	15	54	8×10^{-3}
virD1 (Cre::VirE2)	5	0	16	98	3×10^{-5}
3′virD2 (Cre::VirE2)	43	0	26	34	2×10^{-4}
virD4 (Cre::VirE2)	5	0	18	16	3×10^{-5}
virE2 (Cre::VirE2)	Full	23	19	39	1×10^{-2}
virF (Cre::VirE2)	Full	8	25	72	3×10^{-3}
virD1+pD3, D4 (Cre::VirE2)	Full	13	46	72	3×10^{-3}
3′virD2+pD3, D4 (Cre::VirE2)	Full	11	30	40	4×10^{-3}
B. On NLS::Cre::VirFΔ42N protein translocation from *Agrobacterium* into yeast[b]					
1100 (Cre)	9	0	241	5	4×10^{-6}
1100 (NLS::Cre::VirFΔ42N)	Full	80	74	25	1×10^{-2}
virA (NLS::Cre::VirFΔ42N)	1	0	43	15	2×10^{-6}
virB4 (NLS::Cre::VirFΔ42N)	8	0	89	2 (10^{-5})	9×10^{-6}
virB7 (NLS::Cre::VirFΔ42N)	3	0	53	26	6×10^{-6}
virG (NLS::Cre::VirFΔ42N)	27	0	387	74	7×10^{-6}
virC2 (NLS::Cre::VirFΔ42N)	Full	51	74	12	7×10^{-3}
virD1 (NLS::Cre::VirFΔ42N)	12	0	68	18	2×10^{-5}
3′virD2 (NLS::Cre::VirFΔ42N)	301	0	55	29	5×10^{-4}
virD4 (NLS::Cre::VirFΔ42N)	1	0	104	10	1×10^{-6}
virE2 (NLS::Cre::VirFΔ42N)	Full	26	56	8 (10^{-5})	5×10^{-3}
virF (NLS::Cre::VirFΔ42N)	Full	102	76	18	1×10^{-2}
virE3 (NLS::Cre::VirFΔ42N)	Full	80	81	30	1×10^{-2}
1100+pD3, D4 (NLS::Cre::VirFΔ42N)	Full	106	104	24	1×10^{-2}
virD1+pD3, D4 (NLS::Cre::VirFΔ42N)	Full	131	115	19	1×10^{-2}
3′virD2+pD3, D4 (NLS::Cre::VirFΔ42N)	Full	152	107	12	1×10^{-2}

[a]*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::VirFΔ42N (**B**). All *vir* mutants, except for *virF* and *virE3*, which are deletion mutants, were obtained by Tn*2hoho* insertion. Plasmid pD3,D4 expresses the VirD3 and VirD4 proteins.
[b]The recipient yeast strain contains a *flox*ed *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.
[c]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.
[d,e]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^{-5}.
[f]The Cre-excision efficiency is determined by the number of yeast colonies on medium containing FOA per number of surviving yeast colonies (output yeast).

higher than the background level [LBA1100 (wild type *vir*) expressing only Cre]. This was expected since the VirA/VirG two component regulatory system is responsible for transcriptional activation of all the *vir* genes. Similar low numbers of Ura$^-$ colonies were detected after co-cultivation of yeast with donor strains having a (polar) mutation in *virB4*,

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::VirFΔ42N 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 translocation.

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 co-infection 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::VirFΔ42N from *Agrobacterium* into yeast. Results of a representative co-cultivation experiment are given in Table 4. Translocation of Cre::VirE2 or NLS::Cre::VirFΔ42N 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:VirFΔ42N 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[a]

Agrobacterium strain[b]	No. yeast colonies[c] Not diluted	10^{-2} Dilution	Output yeast[d] 10^{-4}	Output Agro[e] 10^{-6}	Excision efficiency[f]
1100 (Cre)	6	0	201	94	3×10^{-6}
1100 (NLS::Cre::VirFΔ42N)	Full	115	326	76	3×10^{-3}
1100 (Cre::VirE2)	192	1	250	31	8×10^{-5}
1100 (NLS::Cre::VirE3)	295	0	275	85	1×10^{-4}

[a]The recipient yeast strain contains a f*lox*ed *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.
[b]*Agrobacterium* strain LBA1100 (wild type, disarmed pTi) carried a non-mobilisable plasmid expressing Cre alone (negative control), NLS::Cre::VirFΔ42N, Cre::VirE2 or NLS::Cre::VirE3, respectively.
[c]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.
[d,e]The output number of yeast and *Agrobacterium* cells was determined on LC and MY medium, respectively.
[f]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 4. The inhibitory effect of the Osa protein on Cre::Vir protein translocation from *Agrobacterium* into yeast[a]

Agrobacterium strain[b]	No. yeast colonies[c] Not diluted	10^{-2} Dilution	Output yeast[d] 10^{-4}	Output Agro[e] 10^{-6}	Excision efficiency[f]
1100 (none)	1	0	41	23	2×10^{-6}
1100::*cre*	1	0	39	41	2×10^{-6}
1100::*cre::virE2*	416	4	52	29	8×10^{-4}
1100::*cre::virE2* + pOsa	0	0	61	18	$<2 \times 10^{-6}$
2561::*NLS::cre::virFΔ1–126*	Full	37	36	46	1×10^{-2}
2561::*NLS::cre::virFΔ1–126* +pOsa	7	0	71	17	1×10^{-5}

[a]The recipient yeast strain contains a f*lox*ed *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.
[b]*Agrobacterium* 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::virFΔ1–126* expressing NLS::Cre::VirFΔ42N or NLS::Cre::VirFΔ42N + Osa, respectively. The genes were integrated by a single cross over in the Ti-plasmid.
[c]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.
[d,e]The output number of yeast and *Agrobacterium* cells was determined on LC and MY medium, respectively.
[f]The 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 the 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 tumourigenesis 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 *A.tumefaciens* 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 *A.tumefaciens* 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 delivering proteins of interest fused to VirE2, VirE3 or VirF or their transport domain(s) into plants, yeast and fungi.

Most of the techniques to mutate yeast genes are based on homologous recombination events during mitosis (58). Nutritional markers are commonly used as selection markers in yeast (59). However, the availability of different nutritional markers for selection is limited. An alternative is the *kan*^{R} marker, which has been shown to be a good marker in yeast (60). Recently, mutagenesis methods were developed in which sequences homologous to the target site in the yeast genome (61) contain a selection marker flanked by specific target sites (e.g. *lox-kan*MX-*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 TFSS.

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