Agrobacterium infection : translocation of virulence proteins and role of VirF in host cells

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CHAPTER 2

Translocation of the *Agrobacterium tumefaciens* VirD2 and VirE3 proteins into eukaryotic host cells

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ABSTRACT

Agrobacterium tumefaciens uses a type IV secretion system (T4SS) to transfer T-DNA as well as the virulence effector proteins VirE2 and VirF into host cells. Here we used the Cre Reporter Assay for Translocation (CRAfT) in *Arabidopsis thaliana* and yeast cells to analyse whether the Vir proteins VirE1, VirD2 and VirE3 are also translocated. In this assay, translocation of Cre-Vir fusion proteins from bacteria can be detected by a Cre-mediated deletion event in the genome of host cells that results in expression of an antibiotic resistance or visual marker gene. Cocultivation of *A. thaliana* roots containing such 'detector' transgenes, with *A. tumefaciens* expressing the different Cre-Vir protein fusions showed VirB/VirD4-mediated protein translocation of VirE3 and VirD2 into host cells. Further analysis showed that a transport signal is present in the last 50 amino acids of VirE3. However, transport of VirE1 could not be detected. Application of the CRAfT assay in yeast confirmed protein transfer of VirE3. Our findings show that besides the previously reported VirE2 and VirF proteins, VirD2 and VirE3 are directly translocated from *Agrobacterium* into host cells via the VirB/VirD4 transport pore.

INTRODUCTION

Agrobacterium tumefaciens is a soil-born plant pathogenic bacterium that causes crown gall disease on a broad range of plants. A large tumor-inducing (Ti) plasmid from which a DNA segment, the transfer (T)-region, is transferred into host cells during infection is essential for its pathogenicity. Expression of the genes located on the transferred DNA (T-DNA) in the plant leads to the synthesis of plant hormones resulting in tumor formation, as well as the production of opines that are used by the bacterium as a carbon source. The ability of *Agrobacterium* to transfer genetic material to plant cells has been widely used for the genetic transformation of many plant species. Moreover, *A. tumefaciens*-mediated genetic transformation has been reported for other eukaryotic organisms such as yeast (Bundock *et al*., 1995), filamentous fungi (de Groot *et al.,* 1998, Michielse *et al.,* 2004) and mammalian cells (Kunik *et al*., 2001).

Transfer of the T-DNA as well as virulence or effector proteins into host cells depends on the presence of a secretion channel, the type IV secretion system (T4SS), spanning the bacterial envelope. This pore or pilus structure is made up of eleven different VirB proteins and the coupling protein VirD4, which is thought to recruit effector proteins for translocation (Baron *et al,* 2002; Atmakuri, Ding and Christie, 2003). The VirB/VirD4 transport channel of *A. tumefaciens* is the prototype T4SS. The versatile family of T4SSs further includes a large group that is involved in conjugative DNA transfer within and between bacterial species, as well as a group involved in transport of only effector proteins. The latter group is used by a number of human and animal pathogens, such as *Bartonella* spp., *Brucella* spp., *Bordetella pertussis, Helicobacter pylori* and *Legionella pneumophila* (Burns, 1999; Yeo and Waksman, 2004; Llosa and O'Callaghan, 2004). For some of these pathogens the translocated effector proteins have been identified, but it is not known in most cases how these proteins subvert host cellular functions to cause disease (Cascales and Christie, 2003; Nagai and Roy, 2003; Luo and Isberg, 2004; Nagai *et al*., 2005; Schulein *et al.*, 2005).

Several Vir proteins encoded by genes located in the *vir*-region of the Ti-plasmid, as well as some chromosomally (*chv*) encoded proteins (Zupan *et al*., 2000; Susuki, Iwata and Yoshida, 2001) are involved in different steps of the transformation process. The two-component regulatory system VirA/VirG senses plant phenolic compounds released by wounded plant tissue and induces the expression of the *vir* genes (reviewed by Christie, 1997; Hansen and Clinton, 1999; Zupan *et al* 2000). The T-region is flanked by two 23 base pair imperfect border repeats. The relaxase protein VirD2 nicks the DNA in these border regions and by a strand replacement mechanism this leads to the release of a single stranded (ss) linear DNA copy of the bottom strand (T-strand) (reviewed by Mysore *et al.,*1998). The VirD2 protein remains covalently attached to the 5' end of the T-strand and pilots the T-DNA into the host cell.

The *virE* operon encodes three proteins namely VirE1, VirE2 and VirE3 (García-Rodríguez, Schrammeijer and Hooykaas, 2006). VirE2, a ssDNA binding protein, is transported into the plant cell independently of the T-strand (Vergunst *et al*., 2000) and has several functions once inside the host cell (Ward and Zambryski, 2001). VirE2 binds cooperatively to the T-strand and protects it against nucleolytic attack inside the host cell.

This association stabilizes the nucleo-protein complex to promote its transfer into the nucleus (Ziemienowicz *et al*., 2001) while interaction of VirD2, and possibly VirE2, with importins via their nuclear localization sequences, promote the penetration of the T-complex into the nuclear pore (Tzfira *et al.*, 2001). The 7.5 kDa VirE1 protein binds to VirE2 as a chaperone. It was proposed that the interaction with VirE1 avoids self-aggregation of VirE2 and keeps VirE2 in a proper unfolded state for translocation by preventing premature binding to the T-strand or other proteins in the bacterial cell (Deng *et al*., 1999; Sundberg and Ream, 1999; Zhou and Christie, 1999; Sundberg *et al*., 1996). Dumas *et al.* (2001) found that VirE2 can form a transmembrane channel in the plant cytoplasmic membrane that allows transport of anions including DNA molecules. Thus, binding of VirE1 to VirE2 may also protect bacterial membranes from penetration by VirE2. Additional studies have demonstrated that recognition of VirE2 by the translocation apparatus does not depend on the presence of VirE1 in the bacterial cell (Vergunst *et al*., 2003; Atmakuri *et al*., 2003). Frenkiel-Krispin and colleagues suggested that joining of VirE2 subunits to ss-T-DNA could take place inside the VirB/D4 transport channel, with simultaneous release of VirE1 subunits, thus promoting early T-complex formation before entry in the plant cell cytoplasm (Frenkiel-Krispin *et al.*, 2006).

The gene located downstream of the *virE2* gene, *virE3,* encodes a hydrophilic protein of 75.6 kDa. Although its precise function is not yet clear, it was suggested recently that VirE3 is transported into the host cell nucleus where it may induce the expression of genes needed for tumor development (García-Rodríguez, Schrammeijer and Hooykaas, 2006). The VirF protein (22.4 kDa) is an F-box protein (FBP) that is somehow involved in proteolytic degradation of target proteins by the proteasome (Schrammeijer *et al.*, 2001). This thesis describes the identification of putative host target proteins. It has been suggested by others that VirF is involved in proteolytic degradation of a host protein, VirE2 Interacting Protein (VIP1), and thereby indirectly of VirE2, to aid in uncoating of the T-strand (Tzfira, Vaidya and Citovsky, 2004). However, this function for VirF has not yet been confirmed during infection. VirE3 and VirF are both necessary for full virulence on some host plants. *A. tumefaciens* double *virF/virE3* mutants are more strongly attenuated than the single mutants, suggesting that VirE3 and VirF play additive roles during infection (Schrammeijer, 2001, García-Rodríguez, Schrammeijer and Hooykaas, 2006).

VirE2 and VirF are translocated directly into host cells in a VirB/VirD4-dependent way (Vergunst *et al*., 2000). The Cre Reporter Assay for Translocation (CRAfT) assay was developed (Vergunst *et al*., 2000) to directly and efficiently detect translocation of *Agrobacterium* effector proteins into host cells. Briefly, the Cre recombinase is used as a reporter to detect intercellular protein translocation of Vir proteins that are expressed as a protein fusion with Cre in bacteria. Translocation of Cre-Vir fusion proteins can be detected by a Cre-mediated deletion event in the genome of host cells that results in expression of an antibiotic resistance or visual marker gene. Subsequent analysis of VirF indicated that a transport signal is located at the C-terminal part and that this signal has a positively charged consensus (Vergunst *et al.,* 2005). The transport signal is essential for recruitment of effectors by the T4SS. In line with the anticipated role of the VirD4 protein as a coupling protein for recruitment of substrates Atmakuri *et al* (2003) showed co-localization of VirE2

using chemically cross-linked complexes of VirE2 and the coupling protein VirD4, at the *A. tumefaciens* cell poles (Atmakuri *et al*., 2003).

In addition to VirE2 and VirF, other Vir proteins such as VirE1, VirE3 and VirD2, possibly play a role *in planta* during infection. Therefore, we analyzed translocation of VirE1, VirE3 and VirD2 into host cells via the VirB/VirD4 channel using the CRAfT assay in both *Arabidopsis* and yeast. We obtained evidence for translocation of the VirE3 protein into plant and yeast cells. In addition, we delineated a transport domain at the C-terminus of VirE3 that has the same Arg-Pro-Arg motif as that present in the C-termini of VirF and VirE2. This, together with the finding of two putative nuclear localization signals (Schrammeijer, 2001) and interaction with host transcription factors, suggests an important function for VirE3 in the host cell nucleus during tumorigenesis. In addition, using a sensitive *Arabidopsis* reporter plant with GFP as a read-out for translocation we found evidence for translocation of the VirD2 relaxase.

EXPERIMENTAL PROCEDURES

Strains, general culture conditions and plasmid isolation

Bacterial and yeast strains used in this study are listed in Table 1. *Escherichia coli* DH5α and KA817 strains were incubated overnight at 37°C in LC-medium, liquid or agar plates (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract and 8 g/L NaCl, pH 7.0), containing carbenicillin (100 μg/ml) or gentamycin (10 μg/ml). *Agrobacterium tumefaciens* strains containing *cre::vir* fusion plasmids were grown overnight in 5 ml of LC media supplemented with spectinomycin (250 μg/ml) and gentamycin (40 μg/ml) or incubated for two days on agar plates at 29°C. At the onset of the experiments *A.tumefaciens* strain KE1(LBA2575) (Sundberg *et al*., 1996) was used as a *virE1* mutant. Strain KE1 originated from a strain with a total *virE* operon deletion (pTiA6Δ*E*) (McBride and Knauf, 1988). In order to restore the *virE2* gene and obtain a single *virE1* mutant, in this strain, a plasmid named pKC1 was introduced carrying the *virE* operon with a precise in frame deletion of 54 codons (8-62) of the *virE1* gene, but with an intact *virE2* gene. In later experiments we used the deletion mutant LBA2571, which is isogenic to LBA1100, the control strain in our transport experiments, but which has a precise *virE1* deletion (Vergunst *et al*., 2003). Similarly, in initial experiments pPM2260*vir*ΔD2 (GV3101 with helper plasmid pPM2260ΔD2) was used as a source for a precise deletion of *virD2* (in this study named LBA2585). We constructed the precise deletion mutant LBA2556, which is isogenic to LBA1100. *Saccharomyces cerivisiae* strain LBY2 (Schrammeijer *et al*., 2003) was grown overnight in yeast-peptone-dextrose (YPD) medium (Sherman, 1991) at 30°C. DNA manipulation techniques were performed according to Sambrook *et al.,* (1989). Isolation of plasmids (listed in Table 2) was performed using kits (Nucleobond kit-Macherey-Nagel or Qiagen) or according to Birnboim and Doly (1979). Restriction enzymes, T4 DNA ligase and buffers were purchased from New England Biolabs.

Table 1. Strains used in this study

Strains	Description[a]	Reference
Escherichia coli		
DH5α (K12)	F- ϕ80*lac*ZDM15 D(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-	*FOCUS*, 1986. 8:2, 9 Gibco BRL, Breda, The Netherlands
KA817 (GM121)	F⁻ *dam-3 dcm-6 ara-14 fhuA31 galK2 galT22 hdsR3 lacY1 leu-6 thi-1 thr-1 tsx-78*	Jenster *et al*., 1995 Allers *et al.,* 2004
Agrobacterium tumefaciens		
LBA1100	pAL1100 ΔT_L,ΔT_R , Δ*tra*, Δ*occ,* Rif ˙Spc	Beijersbergen *et al*., 1992
LBA1148	pAL1100 (*virD4*::Tn3*Hoho1*), Rif, Cb	Beijersbergen *et al*., 1992
LBA2556	pAL1100 (Δv*irD2*), Rif, Spc	This study
LBA2561	pAL1100 (Δ*virF*), Rif, Spc	Schrammeijer, 2001
LBA2571	pAL1100 (Δ*virE1*), Rif, Spc	Vergunst *et al*., 2003
LBA 2575 (KE1)	pTiA6Δ*E,* Km, pKC1, *virE1*Δ54, *VirE2*, Tc This plasmid harbors a partial sequence deletion of *virE1* (codons 9-62) but restores the full *virE2* sequence.	McBride and Knauf 1988 Sundberg *et al*., 1996
AtΔ*vir*D2	GV3101(pPM6000- Δ*virD2*), Rif,Cb	Bravo-Angel *et al.,* 1998
LBA2585	GV3101, pPM2260, Δ*virD2*, Rif,Cb	Deblaere *et al.,*1985
Saccharomyces cerevisiae		
LBY2	RSY12 containing a *lox* flanked DNA marker (*lox-URA3-lox*) integrated at the *PDA1* locus on chromosome 5	Schrammeijer *et al*., 2003

[a] Rif: rifampicin; Spc: spectinomycin ; Cb: carbenicillin; Km: kanamycin; Tc: tetracycline resistance
Δ: Deletion

Construction of *virD2* deletion mutant LBA2556

Using the marker exchange-eviction mutagenesis method (Ried and Collmer, 1987), a precise deletion mutant of *virD2* was constructed from *A. tumefaciens* LBA1100. To this end, DNA extracted from this strain was used as template to amplify the two flanking sequences of the *virD2* gene, consisting of 441 bp of *virD1* (primers D_2f_2 [5'-CgagctcCCATCAACCAGCATCGATCC-3'; *Sst*I underlined] and D_2r_2 [5'-CGTGAACTGACC ATTTGCCATCCAATTTTCTCCCGTCAG-3']), and 366 bp of *virD3* (primers D_3f_2 [5'-ATGGCA AATGGTTTCACG-3'] and D_3r_1 [5'-CGggatccCTTGCTTGG GAACAGGGAC-3'; *BamH*I underlined]). To perform a fusion PCR lacking the *virD2* gene, both PCR fragments were gel-purified (Qiagen) and mixed in equal amounts to be used as template DNA for the reaction in combination with D_2f_2 and D_3r_1 oligonucleotides. The *virD1-virD3* fusion product was subsequently cloned as *SstI-BamH*I in pGEM-T (Promega Corporation, USA) for sequence analysis, and finally subcloned as *Sac*I-*BamH*I fragment in pSDM3005 (Vergunst *et al*., 2003). The resulting plasmid pSDM3606 was electroporated to *Agrobacterium* strain LBA1100 (den Dulk-Ras and Hooykaas, 1995). Recombination events of Km^r colonies were analyzed as described in Vergunst *et al* (2003), resulting in the LBA2556 mutant strain carrying the precise deletion of the *virD2* gene in the v*ir* region.

Table 2. Plasmids used in this study

Plasmids[a]	Description	Reference
pBI770	GAL4-DNA binding domain bait vector, Cb, *LEU2*	Kohalmi *et al*., 1998
pIC7	Versatile vector in which a synthetic oligonucleotide has been used to replace the pUC8 polylinker and thus to construct a new cloning vector with a different polylinker. The other pIC-vectors are based on this new pIC7 polylinker.	Marsh, Erfle, and Wykes, 1984
pIC19R	Versatile vector based on pIC7 polylinker which was combined with the existing pUC9 and pUC19 polylinkers: *EcoRI*- Poly (pIC7) -*HindIII*- Poly (pUC9) –*EcoRI.*	Marsh, Erfle and Wykes, 1984 http://genome-www.stanford.edu/vectordb/vector_descrip/PIC19R.html
pIC20H	Versatile vector based on pIC7 polylinker which was combined with the existing pUC9 and pUC19 polylinkers: *HindIII*-Poly(pUC19) -*EcoRI* - Poly (pIC7) -*HindIII*	Marsh, Erfle and Wykes, 1984 http://genome-www.stanford.edu/vectordb/vector_descrip/PIC19R.html
pGEM-T	High copy number vector feasible for cloning of PCR products. Contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the alpha-peptide coding region of the enzyme beta-galactosidase.	Promega Corporation, USA
pPG1	Binary plasmid with T-DNA carrying *gus* and *npt*II reporter genes	Vergunst *et al*., 1998
pPM6000	Disarmed pTiAch5 derivative carrying a pBR322 sequence between its TL borders, lacks all TL genes except the octopine synthase gene.	Bonnard *et al*.,1989
pPM2260	Borderless disarmed pTiAch5 derivative	Deblaere *et al.,*1985;
pRL662	pBBR1 MCS ΔmobΔoriT	Vergunst *et al.,* 2000
pRAL3248	pUC19 with fragment *BamH1-1* of pTi15955	Melchers *et al.,*1990
pSDM3005	Non-replicative plasmid in *A. tumefaciens* containing the *Bacillus subtillis sacR/B* gene and Km^r for selection of double crossovers events in *A. tumefaciens.*	Vergunst *et al.,* 2003
pSDM3006	pIC20R, *virE3*	This study Schrammeijer *et al*., 2003
pSDM3043	Binary vector for analysis of Cre recombination events (*pDE35S*[b]*-lox-bar-lox-nptII-ocs*)	Vergunst *et al.,* 2000
pSDM3123	VirE1 and partial virE2 sequences in pIC19R	This study
pSDM3147	pRL662, *pvirE-virE1-cre*	Vergunst *et al.,* 2000
pSDM3155	pRL662, *pvirF-NLS::cre::virFΔ42N*	Vergunst *et al.,* 2000
pSDM3149	pRL662, *pvirD-virD2*	This study
pSDM3189	pUC21, *pvirF-NLS::cre::virF*ΔATG	Schrammeijer *et al*., 2003
pSDM3197	pRL662, *pvirF-NLS::cre*	Vergunst *et al.,* 2000 Schrammeijer *et al*., 2003
pSDM3211	pRL662, *pvirF-NLS::cre::virE3-50C*	Vergunst *et al.,* 2003
pSDM3500	pUC21, *pvirF-NLS::cre::virD2*	This study, Vergunst *et al*., 2005
pSDM3502	pUC21, *pvirF-NLS ::cre ::virE1*	This study
pSDM3503	pBluescriptII KS (+/-), *virE3Δ60N*	This study
pSDM3504	pBluescriptII KS(+/-),*virE3*	This study
pSDM3505	pRL662, *pvirF-NLS::cre::virE1*	This study
pSDM3506	pRL662, *pvirF-NLS::cre::virD2*	This study, Vergunst *et al*., 2005

Table 2. Continuation

Plasmids[a]	Description	Reference
pSDM3507	pRL662, *pvirF-NLS::cre::virE3*	This study Schrammeijer *et al*., 2003 Vergunst *et al.,* 2003
pSDM3606	pSDM3005, *virD1-virD3* fusion	This study
pSDM3639	pBI770, *VirD2*ΔATG	This study, Vergunst *et al*., 2005
pUC vectors	Cloning vectors based on the lac cloning region of the phage vectors M13mp10 and M13mp11.	Norrander, Kempe and Messing, 1983
pVD43	Plasmid containing the *virD* promoter region and the *virD2* structural gene	Rossi, Hohn and Tinland, 1993

[a] The plasmids were electroporated (den Dulk-Ras and Hooykaas, 1995) into *Agrobacterium* (Table 1) as mentioned in the results section.
[b] Promotor region of the 35S transcript of cauliflower mosaic virus with a double enhancer sequence
Km^r: Kanamycine resistance marker

Construction of *virD2* deletion mutant LBA2556

Using the marker exchange-eviction mutagenesis method (Ried and Collmer, 1987), a precise deletion mutant of *virD2* was constructed from *A. tumefaciens* LBA1100. To this end, DNA extracted from this strain was used as template to amplify the two flanking sequences of the *virD2* gene, consisting of 441 bp of *virD1* (primers D_2f_2 [5'-CgagctcCCATCA ACCAGCATCGATCC-3'; *Sst*I underlined] and D_2r_2 [5'-CGTGAACTGACCATTTGCCATCCAA TTTTCTCCCGTCAG-3']), and 366 bp of *virD3* (primers D_3f_2 [5'-ATGGCAAATGGTTTCACG-3'] and D_3r_1 [5'-CGggatccCTTGCTTGG GAACAGGGAC-3'; *BamH*I underlined]). To perform a fusion PCR lacking the *virD2* gene, both PCR fragments were gel-purified (Qiagen) and mixed in equal amounts to be used as template DNA for the reaction in combination with D_2f_2 and D_3r_1 oligonucleotides. The *virD1-virD3* fusion product was subsequently cloned as *SstI-BamH*I in pGEM-T (Promega Corporation, USA) for sequence analysis, and finally subcloned as *Sac*I-*BamH*I fragment in pSDM3005 (Vergunst *et al*., 2003). The resulting plasmid pSDM3606 was electroporated to *Agrobacterium* strain LBA1100 (den Dulk-Ras and Hooykaas, 1995). Recombination events of Km^r colonies were analyzed as described in Vergunst *et al* (2003), resulting in the LBA2556 mutant strain carrying the precise deletion of the *virD2* gene in the v*ir* region.

Construction of Cre recombinase fusion plasmids

cre::virD2 fusion: The construction of a full length *cre::virD2* fusion required several cloning steps. First, an *Eco*RV/*Eco*RI fragment from pVD43 (Rossi, Hohn and Tinland, 1993) containing the *virD* promoter region and the *virD2* structural gene was cloned into *Sma*I/*Eco*RI digested pIC20H (Marsh, Erfle and Wijkes, 1984), then subcloned as a *Sma*I/*Bgl*II fragment into *Bam*HI/*Eco*RV digested pBluescript II SK (Alting-Mees and Short, 1989). A *Pst*I/*Not*I fragment was then cloned into pBI770 (Kohalmi *et al*., 1998) in which a *Sal*I (partial)/*Pst*I (5'-*TCGA*CCCGAT-108 nt *virD2*-GAAC*TGCA*-3') linker had been inserted previously, resulting in pSDM3639. A *Sal*I (partial)/*Not*I fragment from pSDM3639 was inserted into *Sal*I/*Eag*I digested pSDM3189 (Schrammeijer *et al.,* 2003) resulting in pSDM3500. Subsequently, the *virD2* gene was cloned translationally to the 3'-end of *cre* as a

*Sal*I/*Xba*I fragment into pSDM3197 (pSDM3506). pSDM3506 contained the *virF* promoter (p*virF*), the nuclear localization signal (NLS) of the Simian Virus 40 (SV40) virus and the translational fusion *cre::virD2.* The complete cassette is named *pvirF-NLS*::*cre::virD2.*

cre::virE1 fusion: A 2.1 kb fragment from pRAL3248 (Melchers *et al*., 1990) containing the *virE1* and *virE2* sequences was cloned as *Sal*I fragment in pIC19R (Marsh, Erfle, and Wykes, 1984) giving origin to pSDM3123. The *virE1* gene was amplified by PCR from pSDM3123 using primer E1-1 as the 5' primer (5'-acgcgtcgacgtGCCATCATCAAGCCGCATGCG-3'), which contains a *Sal*I site (underlined) and a deletion of the ATG start codon, and E1-2 as the 3' primer (5'-ctagtctaga*TCA*CTCCTTCTGACCAGCAA-3'), which contains an *Xba*I site (underlined) and the termination codon of *virE1* (italics). After *Sal*I-*Xba*I digestion, the PCR product was cloned into similarly digested pSDM3189 (Schrammeijer *et al.,* 2003). The new plasmid, pSDM3502, contained a translational fusion of *virE1* to the 3' end of *cre. Xba*I restriction was tested in the *E. coli* KA817 strain (*dam- dcm-*). pSDM3505 was constructed by cloning a pSDM3502 *Hind*III-*Xba*I fragment into pSDM3197, resulting in p*virF-NLS::cre::virE1*.

cre::virE3 fusion: The *virE3* gene was cloned as an *Eco*RV-*Sac*I fragment from pRAL3248 (Melchers *et al*., 1990) into pIC20R (Marsh, Erfle, and Wykes, 1984) resulting in pSDM3006. The 3' coding region of v*irE3* present in pSDM3006 was subcloned as *Sal*I-*Pst*I fragment into pBluescriptII KS (+/-) (pSDM3503). To remove the ATG start codon, the 5' located 180 nucleotides from *virE3* were amplified by PCR using pSDM3006 as DNA template with primers E3-1 (5'-acgcgtcgac**agatcT**GCGTGAGCACTACG AAGAAAAG-3'), which contained *Sal*I (underlined) and *Bgl*II (bold) sites, and E3-2B (5'-AGCCTATTTCGCCACGAAACCC-3').To reconstruct the complete coding region, the *virE3ΔATG* PCR fragment was digested with *Sal*I and cloned upstream the *virE3* 3' coding region in pSDM3503, resulting in pSDM3504. The *virE3ΔATG* sequence was then cloned as an *Xho*I-*Xba*I fragment into the *Sal*I-*Xba*I sites of pSDM3197 resulting in p*virF-NLS::cre::virE3* (pSDM3507).

cre::virE3-50C fusion: The 150 bp corresponding to the last 50 C-terminal amino acids of VirE3 were cloned behind the *virF promoter-SV40NLS::cre* sequence in pSDM3197 as described by Vergunst *et al.* (2003), giving origin to pSDM3211, or p*virF-NLS::cre::virE3-50C*.

Sequence analysis was performed to confirm the frame and precision of all the *cre::vir* fusions.

Construction of a *pvirD-virD2* plasmid

A fragment containing the *virD* promoter sequence fused to the *virD2* coding sequence (pVD43; Rossi, Hohn and Tinland, 1993) was cloned as *Eco*RV/*Eco*RI fragment in pIC20H and then as *Xba*I/*Eco*RI fragment into similarly digested pRL662. The final vector was named pSDM3149.

Analysis of fusion protein expression by Western blot

A. tumefaciens cells were grown overnight as described above. Cells were inoculated in induction medium (IM) [minimal medium (MM) salts (Hooykaas *et al.,* 1979) plus 40 mM 2-(N-morpholino) ethanesulfonic acid, pH5.3, 10 mM glucose, 0.5% (w/v) glycerol and 200 μM acetosyringone (AS) (Schrammeijer *et al,* 2003)] to a final OD_{600} of 0.2 in the absence of antibiotics. Cultures were incubated overnight at 29ºC and 1.0 ml of cells equal to an OD_{600} of 1.0 was pelleted and resuspended in 120 μl of protein sample buffer (Sambrook *et al.* 1989). Cell lysis was obtained by heating this preparation for 5 min at 100ºC and after elimination of cell debris by centrifugation (13000 rpm, 2 min). Ten μl of each supernatant were loaded onto a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (12%). Immunoblot detection was performed with Cre antibody (1:500) (Eurogentec, Seraing, Belgium) and anti-mouse-alkaline phosphatase conjugate (1:7500) purchased from PROMEGA, Madison, WI U.S.A. Signal detection was obtained using Nitroblue tetrazolium (NBT 50 mg/ml) and 5-bromo-4 chloro-3 indolylphosphate (BCIP 50 mg/ml).

Analysis of recombination activity

A. tumefaciens strains LBA1100(pSDM3505), LBA1100(pSDM3506), LBA1100 (pSDM3507), LBA1100(pSDM3147) and the *virF* mutant strain LBA2561(pSDM3155) were electroporated (den Dulk-Ras and Hooykaas, 1995) with pSDM3043 (Vergunst *et al.,* 2000) and plated on LC containing spectinomycin (250 μg/ml), gentamycin (40 μg/ml) and kanamycin (100 μg/ml). pSDM3043 harbors two *lox* sites and was used in this study to confirm the presence of Cre activity (Vergunst *et al.,* 2000) by deletion of the *lox*-flanked DNA region, visualized by plasmid restriction analysis. Strains containing the different *cre::vir* fusion plasmids in combination with pSDM3043 plasmids were grown overnight in MM with half the usual concentration of spectinomycin and kanamycin. After induction of cultures with AS in IM containing similar antibiotic concentrations as in MM, pSDM3043 DNA was isolated for restriction analysis to detect the occurrence of Cre-mediated deletion.

Protein transport experiments

Plant cocultivation: Cocultivation of root explants of *Arabidopsis thaliana* transgenic line 3043 (Vergunst *et al*., 2000) with *A. tumefaciens* was performed according to Vergunst *et al.* (1998). In some experiments *A. thaliana* line CB1 (Vergunst *et al*., 2005) was used. In this plant line the read-out for protein translocation is GFP instead of kanamycin resistance. Roots were isolated from 10 day-old plantlets cultured in B5 liquid medium and spread on agar plates containing callus induction media (CIM, Valvekens *et al.*, 1988) and incubated during 3 days (25°C/2000 lux). *A. tumefaciens* cells (final OD_{600} of 0.1 in B5) were added to *A. thaliana* root explants and left for two minutes. The explants were dried and cocultivated on CIM containing 100 μM acetosyringone (AS) for two days at 25°C, 3000 lux. Root explants were then transferred to fresh shoot induction media (SIM) (Vergunst *et al.*, 1998) in the presence of 50 mg/l kanamycin and 100 mg/l timentin. The number of kanamycin resistant calli was estimated after 2 weeks of cocultivation.

Yeast cocultivation: *Saccharomyces cerevisiae strain* LBY2 (*lox-URA3-lox*) was co-cultivated with the described *A. tumefaciens* strains according to the method reported by Schrammeijer *et al.* (2003).

Analysis of excision events in plant cells

Shoots derived from kanamycin resistant calli were transferred after three weeks to MA rooting media (Vergunst *et al.*, 1998) and one week later to MS media (Vergunst *et al.*, 1998). Plant DNA was isolated as described by Vergunst and Hooykaas (1998) and primers **a** (5'-GAACTCGCCGTAAAGACTGGCG-3') and **b** (5'-GCGCTGACAGCCGGAACACG-3'), annealing in the *35S* promoter region and the *nptII* sequence respectively, were used for PCR analysis. The conditions were as follows: hot start for 5 min at 96°C, 35 cycles of 1 min 94°C, 1 min 56°C, and 3 min of 72°C, with Ex-Taq polymerase (BioWhittaker, Belgium) as described by the manufacturer.

RESULTS

Cre::Vir translational fusions are expressed in *Agrobacterium tumefaciens*

In order to test whether VirD2, VirE1 and VirE3 are translocated effector proteins, we used the CRAfT assay with *Arabidopsis* line 3043 (Vergunst *et al*., 2000), in which translocation of Cre-Vir fusion proteins from *Agrobacterium* is detected by a Cre-mediated excision of a *lox*-flanked DNA sequence resulting in kanamycin resistance. In addition we used CRAfT in *Saccharomyces cerevisiae* strain LBY2, in which Cre-mediated excision results in resistance to 5-Fluoro-orotic acid (FOA, Apollo Scientific, Ltd., Derbyshire, UK) (Schrammeijer *et al.,* 2003). For this assay, translational fusions of *cre* with *virD2, virE1* and *virE3* were made and electroporated into *Agrobacterium* strain LBA1100 and the *virD2* deletion mutant (pPM2260*vir*∆D2 or LBA 2585) and the *virE1* deletion mutant (KE1 or LBA2575). We performed immunoblot analysis using Cre antibodies to show expression of the translational *cre::vir* fusions in *Agrobacterium* after induction with acetosyringone (Figure 1). All *cre::vir* fusion genes were expressed, and the expected sizes of the fusion proteins were detected (Cre: 38.5 kDa, VirD2::Cre 88 kDa, Cre::VirE1 46 kDa, Cre::VirF 61 kDa, Cre::VirE3 114 kDa and Cre::VirE3-50C 47 kDa). We also tested whether the fusion proteins had Cre recombinase activity. To this end we introduced *lox* substrate plasmid pSDM3043 into the *Agrobacterium* strains with the fusion plasmids. Cre-mediated deletion of the f*lox*ed DNA fragment was analyzed by restriction analysis. This revealed that each of the fusion proteins mediated site-specific recombination at the *lox* sites (data not shown).

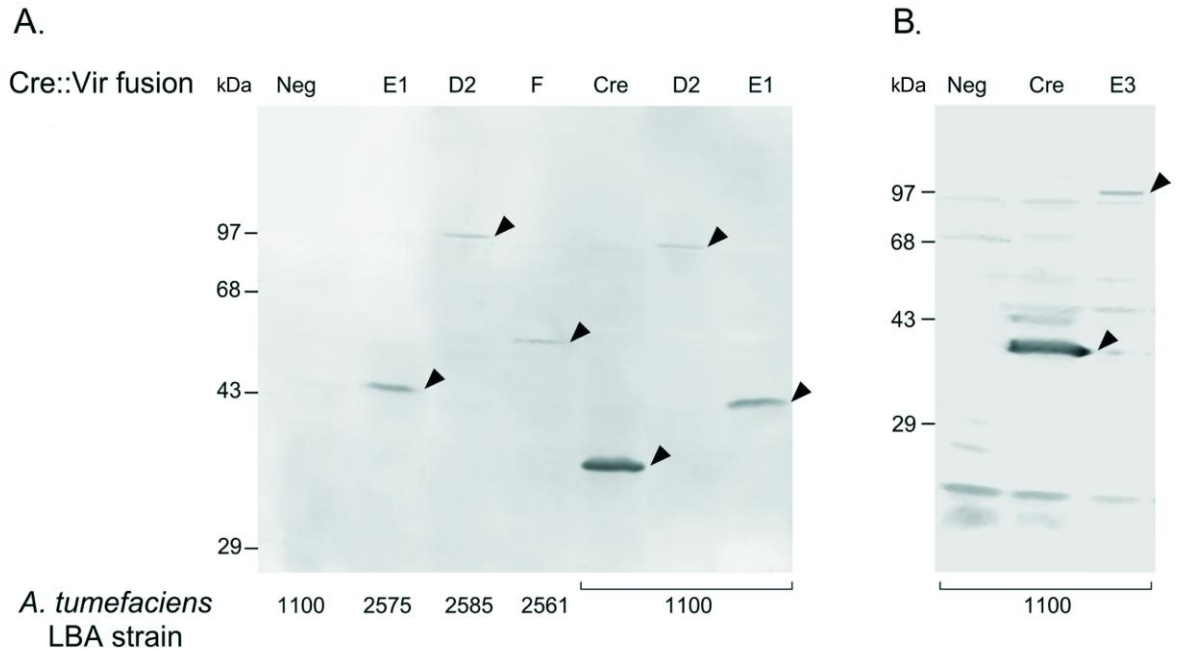

Figure 1. Protein expression of translational Cre::Vir fusions in *A tumefaciens*. (A) Western-blot detection with Cre antibody for Cre::VirE1 (46 kDa), Cre::VirD2 (88 kDa) and Cre::VirF (61 kDa) expressed in LBA1100 and the *vir* mutants LBA 2575, LBA2585 and LBA2561 **(B)** Expression of Cre::VirE3 (114 kDa) in LBA 1100. LBA 1100 with and lacking Cre (38.5 kDa) were used as positive and negative (Neg) controls respectively. Black arrows indicate the expected bands. Neg: strain without Cre fusion protein.

Evidence for translocation of the VirD2 relaxase protein

The VirD2 relaxase binds to the right border sequence of the T-DNA and creates a nick in the bottom strand between nucleotides 3 and 4 (Albright *et al*., 1987; Wang *et al.*, 1987). VirD2 remains attached to the 5' end of the T-DNA (Howard and Citovsky, 1990). It is thought that the VirD2 protein may act as a pilot to transport the T-strand through the VirB/D4 channel (Regensburg-Tuïnk and Hooykaas, 1993), indicating that VirD2 is a transported substrate of the T4SS. In addition, the C-terminus of VirD2 contains an Arg-X-Arg signature that is present in the translocated effector proteins VirE2 and VirF (Vergunst *et al*., 2000). Therefore, we were interested to see whether VirD2 could be directly translocated into host cells. For this we used the Cre recombinase system as a reporter to detect protein translocation into host cells (CRAfT), in which Cre recombinase is expressed in bacteria as a fusion to bacterial proteins to detect their translocation into host cells. To detect protein transport, we used two different *Arabidopsis* reporter transgenic lines, one in which Cre-mediated excision results in expression of a neomycin phosphotransferase (*nptII*) gene, leading to kanamycin resistance (line 3043, Vergunst *et al*., 2000), and one in which Cre activity leads to GFP expression (line CB1, Vergunst *et al*., 2005).We constructed a N-terminal translational fusion of VirD2 to Cre, and expressed this in *A. tumefaciens*. Hence, the *cre::virD2* fusion plasmid (pSDM3506) was introduced into LBA1100 and the *virD2* mutant, LBA2585, both lacking T-DNA. Cocultivation experiments with *A. thaliana* root explants of plant line 3043 resulted in only a few kanamycin resistant calli (0/183 and 1/197

to 3/237 calli/explants respectively) (Table 3). This suggests that Cre::VirD2 may be translocated at a very low efficiency compared to our positive control, Cre::VirFΔ42N (37calli/195explants to 87 calli/183explants) [N-terminal fusion of *VirF* lacking the F-box domain (N-ter 42 aa) to Cre, Vergunst *et al,* 2000)] (Table 3). Although PCR analysis on shoots obtained from these few kanamycin resistant calli showed a 0.7 kb fragment that is expected after Cre-mediated excision at the target locus (Figure 2), we were not able to firmly conclude that VirD2 is transported into host cells in the absence of T-DNA based on this low efficiency. In fact, the excision events could also be due to rare events of homologous recombination (Vergunst e*t al.*, 2000).

Table 3. Transfer of Cre::Vir protein fusions to plant cells

Plasmid	***A. tumefaciens* strain**	**Calli number/total explants[a]**		
		Experiment 1	**Experiment 2**	**Experiment 3**
cre::virD2	LBA1100	2/222 [b]	0/183	ND
	LBA2585 (v*irD2*$^{-}$)	3/237 [b]	1/197	ND
cre::virE1	LBA1100	0/175	ND	ND
	LBA2575 *(virE*$^{-}$ *virE2+)*	0/179	ND	ND
cre::virE3	LBA1100	ND	0/205	10/800
	LBA2575 *(virE*$^{-}$ *virE2+)*	ND	1/197	ND
cre::virE3-50C	LBA1100	ND	ND	124/525
	LBA2571*(virE1*$^{-}$)	ND	ND	99/590
cre::virFΔ42N	LBA1100	ND	ND	612/515
	LBA2571*(virE1*$^{-}$)	ND	ND	390/530
	LBA2561*(virF*$^{-}$)	87/183	37/195	ND
cre	LBA1100	ND	0/147	0/515

[a] The number of kanamycin resistant calli per total number of initial explants (roots) are given.
[b] 0.7 kb PCR amplified fragment obtained after Cre mediated excision of the *lox-bar-lox* segment (Figure 2).
LBA 1100: wild type *vir* loci
ND: not determined

In addition to the plant experiments we tested VirD2 protein translocation into yeast. To this end, the CRAfT assay was applied to *S. cerevisiae* strain LBY2 (Schrammeijer *et al.,* 2003), which contains a genomic *lox-URA-lox* gene for detection of Cre activity. Strains LBA1100 and LBA2585 containing the *cre::virD2* fusion plasmid were cocultivated for six days with LBY2 cells. The efficiency of Cre-mediated *URA3* gene excision as a measure for protein translocation was determined by selection of yeast colonies in the presence of 5-fluoroorotic acid (5-FOA). After cocultivation with *A. tumefaciens* expressing the *cre::virD2* fusion gene we obtained FOA+ colonies at a frequency in the order of 10^{-6} (*URA3* excision/output yeast) similar as the negative control strain expressing only Cre, whereas FOA+ colonies were obtained with an efficiency ranging from 10^{-2} to 10^{-4} after cocultivation with strain LBA1100 (*cre::virFΔ42N*) (Table 4). This is in line with the plant experiments and provides no direct evidence for Cre::VirD2 translocation into host cells.

We were interested to find out whether Cre::VirD2 could functionally complement wild type VirD2. LBA1100 and its full-length *virD2* deletion derivative, LBA2556, were electroporated with the binary plasmid pPG1 (Vergunst *et al.*, 1998) as a source of T-DNA.

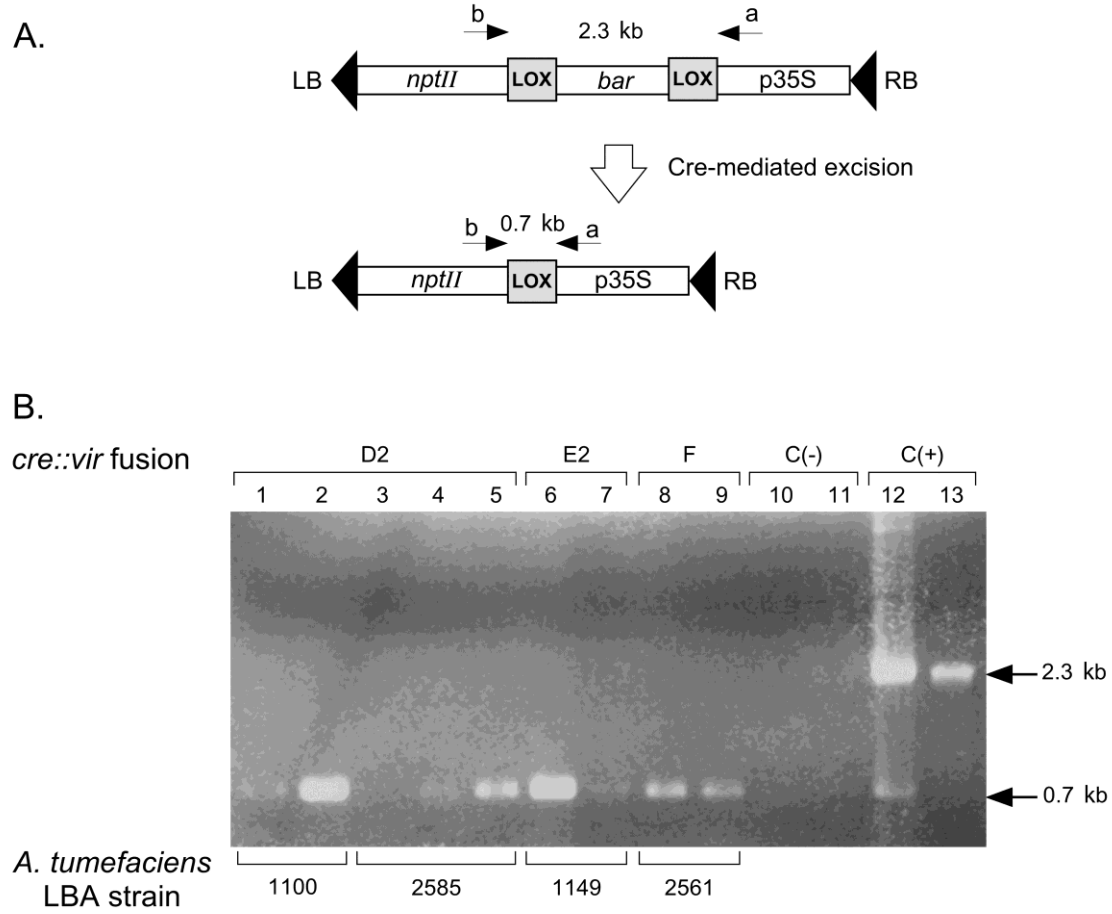

Figure 2. A) Scheme representing Cre-mediated excision in pSDM3043 for reconstitution of *lox-nptII* translational fusion. Primer binding sites are represented as a and b (Vergunst *et al.*, 2000). LB and RB, left and right T-DNA border sequences; *nptII,* neomycin phsophotransferase gene, *bar*, bialaphos resistance gene; p35S, promoter of the 35S transcript of the cauliflower mosaic virus. **B)** PCR analysis with primers **a** and **b** on kanamycin resistant shoots obtained after cocultivation of plant line 3043 with strains containing *cre::virD2*, *cre::virE2* and *cre::virF* fusions. Fragment of 0.7 kb shows Cre-mediated excision on the target *lox* sites. Genomic DNA of wild type *A. thaliana* and transgenic line 3043 were used as negative (C-) (no amplification) and positive (C+) controls (unexcised 2.3 kb fragment) respectively.

This plasmid carries the *npt*II reporter gene on the T-DNA region, allowing selection for kanamycin resistance in plant cells. We used plant line CB1 (Vergunst *et al.,* 2005) for co cultivation experiments. This reporter line contains a GFP gene as read out for protein translocation, and was therefore suitable to detect T-DNA transfer of pPG1 (kanamycin resistance) simultaneously with protein transfer of Cre protein fusions, detected as GFP fluorescence. Co-cultivation of CB1 root explants with LBA1100(pPG1) resulted in large numbers of kanamycinresistant calli as expected (Table 5). Similar large numbers of kanamycin resistant calli were obtained after cocultivation with the derivatives of LBA1100(pPG1) containing pSDM 3197 or pSDM3506. No T-DNA transfer was observed after cocultivation with the *virD2* deletion mutant LBA2556 as expected, because VirD2 is essential for the T-DNA transfer process. Complementation of the absence of VirD2 in this strain with a wild v*irD2* gene resulted as expected in restoration of T-DNA transfer to wild

type levels (LBA2556 (pPG1, 3149) (Table 5). Interestingly, the Cre::VirD2 fusion (pSDM3506) was able to complement the *virD2* defect for T-DNA transfer, although at very low efficiency (2%) (Table 5).

In this cocultivation experiment we were able to assay protein translocation at the same time as T-DNA transfer. Cocultivation of CB1 root explants with LBA1100 (pSDM3155) resulted in large numbers of GFP fluorescing cells, whereas the negative control expressing only Cre protein [LBA1100 (pPG1, 3197)] did not yield any fluorescent cells.

Table 4. Vir protein translocation from *A. tumefaciens* into *S. cerevisiae*[a]

		Frequency *URA-3* excision/output yeast			
Fusion	**Strain**	**Experiment 1**	**Experiment 2**	**Experiment 3**	**Experiment 4**
cre::virD2	LBA1100	6.9×10^{-6}	6.1×10^{-6}	ND	5.9×10^{-6}
	LBA2585	7.2×10^{-6}	ND	ND	
cre::virE1	LBA1100	9.6×10^{-6}	3.8×10^{-6}	ND	ND
	LBA2575	8.2×10^{-6}	ND	ND	
cre::virE3	LBA1100	1.2×10^{-5}	1.1×10^{-4}	1.9×10^{-4}	6.3×10^{-5}
	LBA2575	1.0×10^{-5}	ND	ND	ND
	LBA2571	ND	ND	1.1×10^{-4}	ND
	LBA1148	ND	ND	ND	5.1×10^{-6}
cre::virE3-50C	LBA1100	ND	3.2×10^{-5}	2.0×10^{-4}	2.3×10^{-5}
	LBA2571	ND	ND	2.0×10^{-4}	ND
	LBA1148	ND	ND	ND	4.8×10^{-6}
cre::virFΔ42N	LBA1100	1.8×10^{-4}	3.5×10^{-3}	1.7×10^{-2}	4.0×10^{-4}
	LBA2561	9.5×10^{-5}	ND	ND	ND
	LBA1148	ND	ND	1.3×10^{-5}	ND
Cre	LBA1100	6.3×10^{-6}	2.9×10^{-6}	6.3×10^{-6}	1.2×10^{-6}

[a]The Cre-excision efficiency is determined by the number of yeast colonies on medium containing FOA per number of surviving yeast colonies (output yeast).
ND: not determined

Table 5. Effect of Cre::VirD2 on T-DNA transfer

***A. tumefaciens* LBA strain**	**GFP fluorescence**	**Calli number/total explants**		**Efficiency[a]**		**Percentage[b]**	
		Exp 1	**Exp 2**	**Exp 1**	**Exp 2**	**Exp 1**	**Exp 2**
1100(3155)	++++						
1100(3506)	+						
1100(pPG1)	ND	418/335	282/422	1.25	0.67	100	100
1100(pPG1, 3197)	-	431/480	226/417	0.90	0.54	72	81
1100(pPG1, 3506)	+	439/515	317/398	0.85	0.79	68	118
2556(pPG1)	ND	0/175	0/364	0	0	0	0
2556(pPG1, 3149)	ND	437/380	313/463	1.15	0.68	92	101
2556(pPG1, 3506)	+	8/330	5/330	0.02	0.01	2	1

[a]Data obtained from the ratio kanamycin resistant calli per total number of initial explants (roots)
[b]Ratio from individual efficiencies with LBA1100(pPG1) efficiency.
Strains: LBA 1100: wild type *vir;* LBA 2556: v*irD2* mutant
Plasmids: pSDM3149: *pvirD-virD2*; pSDM3155: *pvirF-NLS::cre::virFΔ42N*; pSDM3197: *pvirF-NLS::cre*; pSDM3506: *pvirF-NLS::cre::virD2;* pPG1: Binary plasmid with T-DNA carrying *gus* and *npt*II reporter genes
ND: not determined

Strains LBA1100(3506), LBA2556(3506), and LBA2556(pPG1, 3506), all expressing the Cre::VirD2 fusion protein, yielded small but reproducible numbers of fluorescing cells (8-10 cell per 200 explants, data not shown). The detection of fluorescent cells was dependent on the presence of a complete T4SS as no fluorescent cells were detected from a *virD4* mutant (data not shown). These data show that the VirD2 protein is translocated into host cells in the absence of T-DNA transfer with low efficiency.

VirE1, the VirE2 chaperone, is not translocated into host cells

VirE1 is a 7.5 kDa protein that is thought to prevent VirE2 aggregation and binding to the T-strand in the bacterial cell. We tested whether VirE1 itself is also a translocated protein. Therefore, a *cre::virE1* translational fusion was introduced in LBA1100 and the *virE1* mutant LBA2575 and thereafter we performed CRAfT experiments with these strains. No kanamycin resistant calli resulted from co-cultivation of the *A. tumefaciens* strains carrying the *cre::virE1* fusion with the *A. thaliana* line 3043 root explants (Table 3). Similarly, cocultivation of LBA1100 and LBA2575 expressing Cre::VirE1 with *S. cerevisiae* LBY2 cells provided no evidence for protein translocation as FOA+ colonies (indicative of *URA3* excision) were obtained with similar frequency as the negative control strain expressing only Cre (10^{-6}) (Table 4). In addition, VirE1 translocation was also not detected using the sensitive reporter *Arabidopsis* line CB1, where no fluorescent cells were seen after cocultivation with LBA1100(pSDM3505) (data not shown). These results indicate that VirE1 is not transferred to plant or yeast cells.

Translocation of VirE3 into host cells requires a functional T4SS

At the onset of these experiments the VirE3 protein was considered as a virulence factor during *Agrobacterium* infection, although its role remained unclear (Schrammeijer, 2001). An Arg-Pro-Arg motif, which was proposed to be part of the transport signal that mediates the translocation of VirE2 and VirF proteins to plant cells (Vergunst *et al*., 2000), is present in the C-terminus of VirE3. This strengthens the hypothesis that VirE3 may be translocated into host cells. To test this, c*re::virE3* fusion plasmids were introduced into *A. tumefaciens* strain LBA1100 and cocultivated with root explants of *A. thaliana* line 3043. Only in one experiment (Table 3) an indication for protein translocation was found, although with low efficiency (10 calli/800 explants). We were interested to find out, whether the C-terminus of VirE3 also contained a transport signal, as thus is the case for VirF and VirE2. Therefore, we tested translocation of a fusion protein consisting of Cre with the 50 C-terminal amino acids of VirE3 (pSDM3211). Cocultivation with LBA1100 expressing the *cre::virE3-50C* fusion resulted in 124 kanamycin resistant calli per 525 root explants, indicating that a transport signal indeed is present in these 50C terminal amino acids.

Similarly, we used the CRAfT assay to analyze translocation of both Cre::VirE3 fusions into yeast cells. After cocultivation of yeast with LBA1100(*cre::virE3*) FOA+ colonies were obtained in two experiments with frequencies of 1.9×10^{-4} and 1.1×10^{-4} (*URA* excision/output yeast). This transfer efficiency is intermediate between the negative control, LBA1100 (*cre*) (6.3×10^{-6}), and the positive control, LBA1100 (*cre::virF*Δ*42N*) (1.7×10^{-2})

(Table 4), and indicates that transfer of full length VirE3 is detected better in yeast than plant cells. Similar frequencies as for full length VirE3 were obtained after cocultivation of yeast with LBA1100 expressing *cre::virE3-50C* ($2X10^{-4}$). This result shows that transport of complete VirE3 and the shorter 50 C-terminal region was detected at similar frequencies in yeast. The difference in detection of transfer of full length Cre::VirE3 into plants and yeast may depend on interactions with host factors that interfere with the detection of the full length Cre::VirE3 fusion in plant but not yeast cells.

We were interested to know if the VirE1 protein is required for efficient transfer of VirE3. Therefore, we introduced the *cre::virE3* and *cre::virE3-50C* plasmid in *virE1* mutant LBA2575 and LBA2571. After cocultivation with *Arabidopsis* 3043 root explants we detected 1 Km^r callus per 197 root explants for LBA2575 (*cre::virE3*), and 99 Km^r calli per 590 explants for LBA2571 (*cre::virE3-50C*) (Table 3). These data are comparable to the results obtained with LBA1100, carrying a wild type *vir* region. After cocultivation with LBY2 yeast cells we obtained similar transfer efficiencies of Cre::VirE3 and Cre::VirE3-50C from a *virE1* mutant and from LBA1100 (Table 4). Experiments with both hosts clearly indicate that VirE1 is not essential for transport of VirE3.

Since transport of VirE2 and VirF requires a functional VirB/VirD4 transport pore, we determined the translocation efficiency of Cre::VirE3-50C in the absence of VirD4 by expressing the *cre::virE3-50C* fusion in a *virD4* mutant strain (LBA1148). After cocultivation with plant (data not shown) and yeast cells (Table 4), no transfer was observed, showing that the VirB/VirD4 transport channel is crucial for the transfer of VirE3 to host cells.

DISCUSSION

Type IV secretion systems (T4SS) are protein complexes spanning the bacterial envelope that mediate the transport of proteins and/or macromolecular structures into host or recipient cells. A subfamily of T4SS is used for plasmid conjugation to recipient bacteria. In addition, pathogenic bacteria have evolved T4SS to survive in eukaryotic hosts by delivering virulence or effector proteins directly into host cells where these subvert host cell biology in favor of the pathogen. The prototype *A. tumefaciens* T4SS is involved in the transfer of T-DNA as well as transport of virulence proteins such as VirE2 and VirF directly into eukaryotic cells (Regensburg-Tuïnk and Hooykaas, 1993; Vergunst *et al*., 2000; Schrammeijer *et al*., 2003; reviewed by Zupan *et al*., 2000; Cascales and Christie, 2003). It was shown that translocation of the effector proteins VirE2 and VirF was independent of T-DNA transfer. Several other Vir proteins possibly play a role inside the host cell during infection. In this study we used the CRAfT assay to analyze whether the *A. tumefaciens* virulence proteins VirD2, VirE1 and VirE3 are also translocated into host cells (Vergunst *et al*., 2000; 2003; Schrammeijer *et al.,* 2003).

In the CRAfT assay (Vergunst *et al*., 2000) Cre recombinase is expressed in bacteria as a fusion to bacterial proteins to detect their translocation into host cells. After translocation, an SV40 NLS ensures nuclear delivery of Cre. In the nucleus it activates the expression of a selectable (kanamycin resistance in *Arabidopsis* line 3043) or visible (GFP in *Arabidopsis* line

CB1) marker by excision of a *lox*-flanked DNA sequence. In yeast strain LBY2 excision of a *lox*-flanked *URA3* gene results in 5-FOA resistance. Here, we constructed N-terminal translational fusions of the *A. tumefaciens* virulence proteins VirD2, VirE1, VirE3, and the last 50 C-terminal amino acids of VirE3 to Cre, and expressed these in *A. tumefaciens*. Western blot analysis and recombination assays showed that all protein fusions were expressed and displayed functional Cre activity. In transport assays to *Arabidopsis* we were not able to detect reproducible levels of full-length Cre::VirE3 protein transfer. However, we detected transport of the last 50 C-terminal amino-acids of VirE3 fused to Cre with an efficiency of about 20% of that of Cre::virF transfer. Using yeast as a host, we detected translocation of the Cre::VirE3-50C fusion as well as full length Cre::VirE3 with similar efficiency and at significantly higher levels than the negative control expressing only Cre. The transfer of Cre::VirE3 was dependent on a functional T4SS, as no transfer was detected from a *virD4* mutant. These data show that, in addition to VirF and VirE2, VirE3 is translocated into host cells by the T4SS, and that a transport signal is located in the C-terminus. A short C-terminal part of VirE2 and VirF had been shown previously to be sufficient for translocation into plant as well as yeast cells. A close view of this C-terminal transport signal revealed the presence of a common C-terminal Arg-Pro-Arg motif (Vergunst *et al*., 2000; Schrammeijer *et al.,* 2003) that is also present in the C-terminus of VirE3. A detailed mutational analysis of the C-terminal 20 amino acid transport signal of the VirF protein and comparison with sequence profiles of other *Agrobacterium* T4SS effector proteins resulted in the proposition of a transport consensus signal that is positively charged and has a specific hydropathic profile (Vergunst *et al*., 2005). We do not have a clear explanation for the contrasting results with which transport of the full length VirE3 fusion is detected in plant and yeast cells. Similar observations were made for the VirF protein: transport of a shorter version of the protein, devoid of the specific N-terminally located F-box, was more efficiently detected in plants than the full length protein, a difference that was also not detected in yeast (Vergunst *et al*., 2000; Schrammeijer *et al.,* 2003). It was suggested that interaction of the F-box domain with plant proteins (Schrammeijer *et al.,* 2001) may sequester the VirF protein and prevent its entry into the nucleus, and thereby its detection in the CRAfT assay. VirE3 was shown to interact with several host proteins (García-Rodríguez, Schrammeijer and Hooykaas, 2006; Lacroix *et al*., 2005) which may prevent nuclear uptake of the Cre::VirE3 fusion protein, similarly preventing detection of its transport. A difference in yeast and plant proteins interacting with VirE3 may explain the observed difference in detection of transport.

The *A. tumefaciens* VirE1 protein is a chaperone of VirE2. VirE1 is described to prevent the self-aggregation of VirE2, enhance its stability and maintain VirE2 in a translocation competent state (Deng *et al*., 1999; Sundberg and Ream, 1999; Zhou and Christie, 1999). *VirE1*, *virE2* and *virE3* are part of one operon (García-Rodríguez, Schrammeijer and Hooykaas, 2006). To determine whether VirE1 is involved in transport of VirE3, we tested whether a Cre::VirE3-50C fusion could be transported in the absence of VirE1. *VirE1* mutant LBA2571 was equally efficient in translocation of a Cre::VirE3-50C fusion as LBA1100 (Wt Vir) expressing the same fusion (This study, Vergunst *et al*., 2003). This shows that VirE1 is not necessary for transport of VirE3. This result is in line with data

that showed that VirE1 is not essential for recruitment of VirE2 by the T4SS (Vergunst *et al.,* 2003, Atmakuri *et al.,* 2003). Next, we explored whether VirE1 could be a translocated effector protein itself. Transport assays, however, both to yeast and plants gave no indication for translocation of a Cre::VirE1 fusion protein. This is not surprising as a C-terminal consensus transport signal, established for effectors of the VirB/VirD4 T4SS previously (Vergunst *et al.,* 2005), is absent in VirE1.

Recruitment of T-DNA and effector proteins to the transport pore is one of the early events in *A. tumefaciens* infection. The T4SS is build up of 11 VirB proteins and the VirD4 coupling protein that together form the transport channel spanning the bacterial envelope. VirD4 is an inner membrane protein forming the cytoplasmic interface of the transport system. It was proposed that the VirD4 protein might couple proteins involved in DNA processing to the transport machinery (Beijersbergen *et al.,* 1992). The *A. tumefaciens* VirD2 relaxase protein binds to the T-DNA border repeat sequences, and cleaves the strand that is destined for transfer in a process reminiscent of conjugative DNA processing T4SS. VirD2 remains attached to the 5'-end of the T-DNA strand, and is thought to pilot the T-strand to the transport pore and into the host cell. Here we showed using CRAfT and a highly sensitive GFP reporter plant line that a Cre::VirD2 fusion protein is transported into plant cells both in the presence and absence of T-DNA (Vergunst *et al*., 2005). Transport was not detected in the absence of VirD4 showing that a functional T4SS is required. This suggests that VirD2 carries a transport signal that is recognized by the T4SS, and that recognition and recruitment of the T-DNA by the T4SS is mediated by the VirD2 protein. With an elegant method using transfer DNA immunoprecipitation assays (TrIP), Cascales and Christie (2004) demonstrated that VirD4 is the first T4SS subunit of an ordered substrate transport pathway of the VirD2/T-DNA complex. Our findings are also in line with results reported for MobA, the relaxase of the RSF1010 IncQ plasmid. It was recently shown that the Dot/Icm T4SS of *Legionella pneumophila* mediates MobA translocation to *E. coli* (Luo *et al*., 2004), which is in agreement with the observations by Vergunst *et al*. (2005) who demonstrated DNA-independent MobA transfer to plant cells via the *A. tumefaciens* VirB/VirD4 pore (Vergunst *et al*., 2005). Together, there is increasing evidence that conjugation systems are actually protein transport systems, in which the relaxase protein is recruited by the coupling protein and translocated into recipient cells, thereby taking the DNA along as a passenger (Regensburg-Tuïnk and Hooykaas, 1993; Vergunst *et al* 2000; Cascales and Christie, 2004).

With our finding that Cre::VirD2 transport into plant cells was detected at low efficiency, we analyzed whether Cre::VirD2 could complement an *A. tumefaciens virD2* mutant for T-DNA transfer. Cocultivation of *virD2* mutant LBA2556, carrying a T-DNA vector with a kanamycin resistance gene and expressing Cre::VirD2, with *Arabidopsis* root explants resulted in kanamycin resistant calli at an efficiency 1 to 2 % of that after cocultivation with wild type *A. tumefaciens* or a *virD2* mutant expressing wild type VirD2 protein. This suggests that Cre::VirD2 can complement wild type VirD2 function with low efficiency. VirD2 remains attached to the T-DNA via a tyrosine-linkage at position 29 (Pansegrau *et al*., 1993b) and mutation of this residue abolishes nicking activity (Vogel en Das, 1992). In addition, two histidine residues located nearby Tyr29 are present for coordinated interaction with the Tyr

residue (Vogel *et al*., 1995). VirD1 and VirD2 are both required *in vivo* for the nicking reaction (reviewed by Zupan *et. al.*, 2000). A low efficiency of complementation of VirD2 by Cre::VirD2 in our experiments can therefore be explained by steric hindrance of Cre, fused to the N-terminus of VirD2, or an incorrect conformation of VirD2, resulting in reduced binding and nicking activity of Cre::VirD2 compared to VirD2. However, the C-terminus of VirD2 is 'free' to interact with the transport pore, allowing its translocation into host cells. In addition, relevant domains for nuclear delivery and preciseness of chromosomal integration, NLS and ω sequence respectively (Mysore *et al*., 1998) are located at the C-terminus and are less likely to be affected by the Cre fusion. Summarizing, our data show that both VirE3 and VirD2 are translocated effectors of the *A. tumefaciens* T4SS. The finding that VirD2 can be transported in the absence of T-DNA supports the current idea that it is the relaxase protein bound to the DNA that carries the transport signal for recognition by the T4SS, and that conjugation systems are actually dedicated protein transport systems.

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