Plant Molecular Biology **55:** 531–539, 2004.

Agrobacterium tumefaciens-mediated transformation of plants by the pTF-FC2 plasmid is efficient and strictly dependent on the MobA protein

Thabani Dube[1,†], Igor Kovalchuk[2], Barbara Hohn[3,*] and Jennifer A. Thomson[1]
[1]*Department of Molecular and Cell Biology, University of Cape Town, Private Bag Rondebosch, 7701, South Africa;* [2]*Department of Biological Sciences, University of Lethbridge, 4401 University Drive, Lethbridge, Alberta TIK 3M4, Canada;* [3] *Friedrich Miescher Institute for Biomedical Research, PO Box 2543, CH-4002 Basel, Switzerland (*author for correspondence; e-mail barbara.hohn@fmi.ch)*

Received 26 April 2004; accepted in revised form 6 July 2004

Key words: *Acidithiobacillus ferrooxidans* plasmid, *Agrobacterium tumefaciens*, border sequence, origin of transfer, plant transformation, T-DNA

Abstract

In the transformation of plants by *Agrobacterium tumefaciens* the VirD2 protein has been shown to pilot T-DNA during its transfer to the plant cell nucleus. Other studies have shown that the MobA protein of plasmid RSF1010 is capable of mediating its transfer from *Agrobacterium* cells to plant cells by a similar process. We have demonstrated previously that plasmid pTF-FC2, which has some similarity to RSF1010, is also able to transfer DNA efficiently. In this study, we performed a mutational analysis of the roles played by *A. tumefaciens* VirD2 and pTF-FC2 MobA in DNA transfer-mediated by *A. tumefaciens* carrying pTF-FC2. We show that MobA+/VirD2+ and MobA+/VirD2− strains were equally proficient in their ability to transfer a pTF-FC2-derived plasmid DNA to plants and to transform them. However, the MobA−/VirD2+ strain showed a DNA transfer efficiency of 0.03% compared with that of the other two strains. This sharply contrasts with our results that VirD2 can rather efficiently cleave the *oriT* sequence of pFT-FC2 *in vitro*. We therefore conclude that MobA plays a major VirD2-independent role in plant transformation by pTF-FC2.

Introduction

Agrobacterium tumefaciens transforms plant cells by transporting DNA, mobilized from a tumor-inducing (Ti) plasmid located in the virulent bacterium, into the plant cell nucleus where it integrates into chromosomal DNA. Expression of this transferred DNA (T-DNA) leads to the formation of crown gall tumors in many dicotyledonous plants. The Ti plasmid also contains the virulence region, which provides several gene products that mediate transformation. The initiator of DNA processing, VirD2, in conjunction with VirDl, cleaves the bottom strand at the 25-bp border repeats and remains covalently attached to the 5′-end of the released strand. This DNA–protein complex is then transferred to the plant cell. VirE2, by binding to the single-stranded T-strand, has been shown to protect the T-DNA from nucleolytic attack. However, successful T-DNA transmission does not require that T-strands and VirE2 protein enter plant cells as a complex. VirE2 was also proposed to cause the formation of pores or channels in plant membranes through which the T-DNA can pass (Dumas *et al.*, 2001; for reviews see Hansen and Chilton, 1999; Gelvin, 2000; Tzfira and Citovsky, 2002).

It has long been known that processing and transfer of the T-DNA region to plant cells shares structural and functional characteristics with

[†] Deceased 12th December 2002.

events occurring during plasmid conjugation (Stachel and Zambryski, 1986; Lanka and Lessl, 1994). For example, the highly conserved T-DNA border sequences are the functional equivalents of the *oriT*, acting as recognition and cleavage sites for the site-specific nicking complexes (Yanofsky *et al.,* 1986; Wang *et al.,* 1987). In addition, the cleaving molecule remains covalently attached to the 5′-end (Dürrenberger *et al*., 1989; Howard *et al*., 1989). Furthermore, both transfer processes involve single-stranded DNA transfer intermediates (Cohen *et al*., 1986; Stachel *et al*., 1986; Albright *et al*., 1987) and both require cell-to-cell contact (Ream, 1989).

It has been shown that the mobilizable IncQ plasmid, RSF1010, requires three proteins for its conjugal mobilization: MobA, which cleaves and ligates the transferred strand (Bhattacharjee and Meyer, 1991) and the two accessory proteins, MobB and MobC. MobB stabilizes the complex of Mob proteins at *oriT* and also has an additional function in the transfer (Perwez and Meyer, 1999). MobC assists in localized separation of the DNA strands at *oriT* (Zhang and Meyer, 1997). In addition, RSF1010 encodes three replication proteins (Scherzinger *et al*., 1984), a helicase, a primase and an iteron-binding protein, the products of the *repA*, *repB* and *repC* genes, respectively.

Derivatives of RSF1010 that carry cognate *mob* genes can be mobilized to plant cells by the Ti plasmid-encoded virulence system (Buchanan-Wollaston *et al*., 1987). Bravo-Angel *et al*. (1999) demonstrated that the RSF1010 MobA-mediated DNA mobilization by *Agrobacterium* to plants was about 100-fold less efficient than that mediated by VirD2.

We have shown previously that a derivative of the plasmid pTF-FC2, like RSF1010 derivatives, is able to transfer DNA to plants without the use T-DNA borders (Dube and Thomson, 2003). pTF-FC2 is a 12.2-kb, non-conjugative, broad host-range plasmid that was originally isolated from the biomining bacterium *Acidithiobacillus ferrooxidans* (previously called *Thiobacillus ferrooxidans*) (Rawlings *et al*., 1984). The plasmid was found to be unique in that it is a hybrid consisting of an IncQ-like replication region and an IncP-like mobilization region (Rohrer and Rawlings, 1992; Rawlings and Tietze, 2001).

The gene arrangement in pTF-FC2 is similar to the ones described for RSF1010 and RP4 (Dorrington *et al*., 1991; Rohrer and Rawlings, 1992). Although the mobilization regions are different at the sequence level the similarities at the functional level are remarkable. The only notable difference is that the three critical proteins for mobilization in pTF-FC2 are MobA, MobC, and MobD. MobB and MobE were found to affect the frequency of mobilization. It is also interesting to note that the region in which pTF-FC2 changes from IncP-like to IncQ-1ike lies within the large continuous MobA-RepB reading frame (Rohrer and Rawlings, 1992).

Since in the previous work both the pTF-FC2 encoded mobilization functions and the Ti plasmid-encoded VirD2 protein were present in the agrobacterial strain used (Dube and Thomson, 2003) we here analyze the contribution of each protein to DNA transfer and integration. *Agrobacterium* strains were constructed that lacked one or the other function and tested for transfer and integration of test-genes located on the pTF-FC2 plasmid. In parallel, *in vitro* cleavage activities of VirD2 on single-stranded DNA fragments containing T-DNA border or the pTF-FC2 mobilization origin were tested.

Materials and methods

Molecular methods

Unless otherwise stated all DNA manipulations and bacterial media preparations were performed according to Sambrook and Russell (2001). Sequencing was done using the ALFexpress DNA automated sequencer (Amersham Pharmacia Biotech AB, Uppsalla, Sweden). The thermo sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP was used according to the manufacturer's instructions (Amersham Pharmacia Biotech).

In vitro *cleavage by VirD2 of a synthetic oligonucleotide containing the* oriT *region of pTF-FC2 or the T-DNA border region*

The VirD2 protein was over-expressed in *Escherichia coli* and purified by FPLC (Pansegrau *et al*., 1993). The oligodeoxyribonucleotides GGTATATATCCTGCCAGTCTTGATGCCGC GCAT, derived from the right border of the

octopine-type plasmid pTiA6, and AACGGTC ATCCTGTATTGCTCAACCGCTCTACTATC ATATC, derived from the *oriT* of pTF-FC2, were used to test the *in vitro* activity of VirD2. 5′-end labelling was performed using (γ^{32}P) ATP (Amersham, Little Chalfont, UK) and T4 polynucleotide kinase (Boehringer Mannheim, Germany). *In vitro* cleavage was performed as described by Pansegrau *et al*. (1993). A concentration of 5 μg of VirD2 was used. Oligonucleotides were incubated at 37 °C with VirD2 for 3 h in 20 μl reaction buffer (20 mM Tris-HCI, pH 8.5, 5 mM $MgCl_2$, 50 mM NaCl). The reaction products were separated on a 20% polyacrylamide gel containing 8 M urea.

Quantification of radioactive signal

Autoradiography was quantified using a PhosphoImager device and IMAGEQUANT software version 3.3 (Molecular Dynamics, Sunnyvale, CA, USA).

Construction of pDER-bar-*GUS*

The construction of plasmid pDER-*bar*-GUS was done by the introduction of the *Eco*RI fragment of pGUS23 (Puchta and Hohn, 1991) that contained both the GUS gene (*uidA*) driven by the 35S Cauliflower Mosaic Virus promoter and the nopaline synthase terminator into the *Eco*RI site of plasmid pDER-*bar* (Figure 3), the construction of which was described (Dube and Thomson, 2003).

*Construction of pDER-*Δ*MobA*-bar-*GUS*

In order to create an internal deletion within the *mobA* gene two divergent primers, MobΔAF and MobΔAR were used to amplify the rest of the plasmid pDER-*bar*-GUS. This was achieved by making use of the Expand Long Template PCR kit according to the manufacturer's instructions (Roche, Mannheim, Germany). Ten nanogram of plasmid DNA were used as template. The cycle conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 20 cycles at 94 °C for 20 s, 64 °C for 30 s and 68 °C for 15 min with an additional 20 s added after each cycle. The final cycle at 68 °C had an elongation time of 7 min. The Polymerase chain reaction (PCR) product that was generated was cloned into the pGEM®-T Easy vector in accordance with the manufacturer's method (Promega, USA). The resultant plasmid, pGEMΔMobA was sequenced to confirm the precision of the deletion. After sequencing, the fragment containing the pGEM®-T Easy vector was excised with *Not*I and discarded. The remaining fragment consisting of the pDER-*bar*-GUS plasmid that contained the *mobA* deletion was purified from the agarose gel using the High Pure PCR Product Purification Kit (Roche). This fragment was self-ligated, resulting in plasmid pDER-*bar*-GUS-ΔMobA in which 1084 bp of the 1228-bp open-reading frame of *mobA* were deleted. The plasmids were introduced into *Agrobacterium* using electroporation.

A. tumefaciens *strains used*

In order to study the roles played by mobA and virD2 an *A. tumefaciens* strain devoid of virD2, GV3101 (pPM6000K), and GV3101 (pPM6000), which had a functional and intact *virD2* gene, were used. Strain GV3101 (pPM6000), a cured C58 nopaline strain containing the pTiAch5 derivative pPM6000, which is deleted in the T-DNA (Bonnard *et al*., 1989) was modified in the *virD2* gene of the Ti plasmid, yielding strain GV3101 (pPM6000K). The latter strain, due to a large in-frame *virD2*-internal deletion, lacks 70% of the coding sequence of the VirD2 protein, which is essential for T-DNA transfer. This strain was shown to be transfer-defective (Rossi *et al*., 1993a). A summary of the strains used is given in Table 1.

Co-cultivation with tobacco cotyledons

The co-cultivation experiment was performed essentially as described by Rossi *et al*. (1993b).

Table 1. Agrobacterium strains used in this study.

Strains	Relevant genotype	
	*vir*D2	*mob*A
GV3I01 (pPM6000)(pDER-*bar*-GUS)	+	+
GV3101 (pPM6000K)(pDER-*bar*-GUS)	−	+
GV3I01 (pPM6000)(pDER-*bar*-GUS-Δ*Mob*A)	+	−
GV3I01 (pPM6000K)(pDER-*bar*-GUS-Δ*Mob*A)	−	−

About 400 *Nicotiana tabacum* SRI seedlings (2 weeks old) were incubated for 3 days with the following *A. tumefaciens* strains: GV3101 (pPM6000)(pDER-*bar*-GUS), GV3101 (pPM6000) (pDER-*bar*-GUS-ΔMobA), GV3101 (pPM6000K) (pDER-*bar*-GUS), and GV3101 (pPM6000K) (pDER-*bar*-GUS-ΔMobA). The non-transformed strain GV3101 (pPM6000K) was used as a negative control. A 5 ml overnight culture of *A. tumefaciens* grown in YEB medium containing the antibiotics rifampicin (100 mg/ml) and kanamycin (100 mg/ml) was washed twice with an equal amount of 10 mM $MgSO_4$ and resuspended in 10 ml MS medium (Murashige and Skoog, 1962) to a final optical density of 0.6 at 600 nm. About 200 seedlings were added to the bacterial suspension. The mixture was exposed to a reduced pressure (0.15 atm) in a sterile vacuum chamber for 5 min. The seedlings were later placed on MS plates (1% agar) and further co-cultivated for 3 days in a growth chamber at 25 °C with 16 h light/day. The plantlets were washed in sterile 10 mM $MgSO_4$ and blotted dry in a sterile vacuum chamber for 5 min. Transient expression of *uidA* was determined by measurement of *β*-glucuronidase activity *via* a histochemical assay using 5-bromo-4-chloro-3-indolyl-glucoronide (X-glu), as described (Rossi *et al.,* 1993b). After co-cultivation, plants were added to 5 ml of GUS staining solution. A soft vacuum of about 0.15 atm was applied for 5 min in order to standardize the diffusion of the substrate into the cells of different tissues in the plantlets. The reaction was allowed to proceed for 24 h at 37 °C. The plantlets were washed in water and bleached with ethanol. The blue sectors present on the plantlets were counted under a microscope. The remaining 200 seedlings were analyzed for T-DNA integration. The seedlings were washed with 10 mM $MgSO_4$ and placed on MS medium containing 0.1 mg/ml naphtylacetic acid, 1 mg/ml benzylamino purine, 20 μg/ml phosphinotricin, 500 mg/ml vancomycin, and 500 mg/ml claforan. The seedlings were transferred to fresh plates every week. Calli were counted after 6–8 weeks.

Results

The results from our previous study in which we demonstrated that a vector derived from plasmid pTF-FC2, pDER-*bar*, could mediate its transfer to plant cells using the T-DNA transfer machinery of *A. tumefaciens* (Dube and Thomson, 2003) raised a number of questions. One pertained to the mechanism of action that enabled such a transfer in general and most importantly the specific roles played by VirD2 and MobA proteins. It was not clear whether the protein that was responsible for the recognition and nicking of the *oriT* region of pTF-FC2 plasmid and its subsequent transfer to plants was VirD2 or MobA or both. In order to determine the contribution of each of these two proteins in this transfer process we investigated their ability to cleave the *oriT* sequence and to transfer plasmid DNA into plant cells. Thus, we determined the transfer of an *oriT*-containing plasmid mediated by pTF-FC2 MobA in a VirD2-free *Agrobacterium* strain. In parallel, transfer of the same plasmid by a *virD2* containing strain but lacking the *mobA* gene was essayed.

Since *in vitro* cleavage of substrate DNA by VirD2 protein has been documented (Pansegrau *et al.,* (1993), we also tested the activity of this protein to cleave the pTF-FC2 origin of transfer.

Sequence alignments of relaxases and T-DNA borders/origins of transfer of mobilizable plasmids

Since the aim of this study was to determine the mechanism of action of DNA transfer to plants by pTF-FC2 it was necessary to establish the evolutionary relationship of the cleavage proteins from various bacteria. Of particular interest was the relationship between VirD2 and the MobA proteins of pTF-FC2 and RSF1010 which was analyzed using CLUSTAL X (Version 1.81) and MACBOXSHADE (Version 2.15) (Figure 1). Sequence-function analyses of the relaxase Tra1 of RP4 had revealed three distinct domains which were found to be conserved in other relaxases such as VirD2 and MobA of pTF-FC2 (Pansegrau *et al.*, 1994). Although some amino acids are conserved in these domains in all three sequences, MobA of RSF1010 shows a lower degree of conservation in these motifs. Especially low is the identity/similarity in motif II, which has been suggested by Pansegrau *et al.* (1994) to represent the DNA recognition domain of Tra1 and related relaxases. Indeed, the recognition sequence for the RSF1010 relaxases at *oriT* is different from that of the IncP-like plasmids.

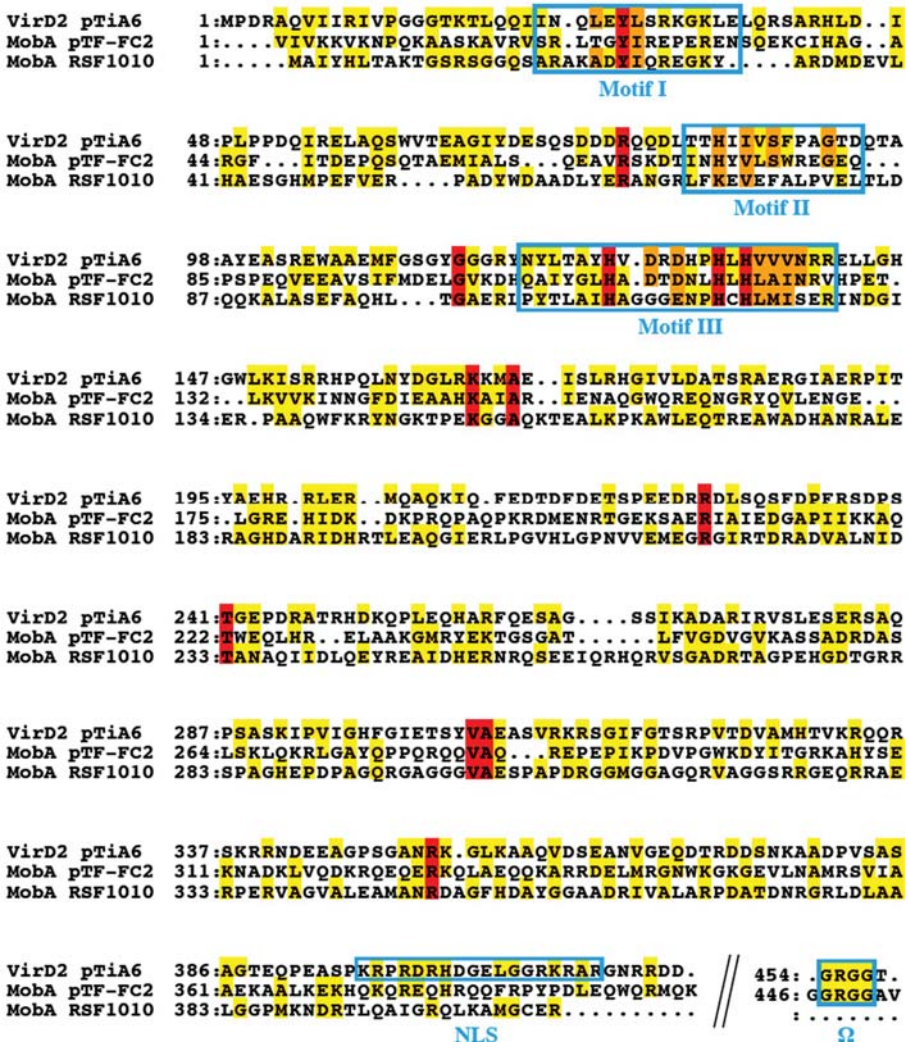

Figure 1. Alignment of the amino acids of VirD2, MobA of pTF-FC2 and MobA of RSF 1010. Accession numbers are AAA98390.1 for pTiA6, AAA27389.1 for MobA of pTF-FC2 (starting at position 58; for complete DNA sequence the accession number is M57717) and P07112 for MobA of RSF1010. Conserved amino acids are in red, similar amino acids in the conserved boxes are in orange (conservation as defined by Pansegrau *et al.*, 1994) and other similar amino acids are in yellow.

It should be noted that the sequence used for the pTF-FC2 MobA protein is that published by Pansegrau *et al.* (1994) (with the exception of the first amino acid which is expected to be a valine, not methionine) and not that of Rohrer and Rawlings (1992). The latter sequence is shorter by 58 aminoacids at the N-terminus and does not contain motif I, an absolutely essential hallmark of a relaxase. Also the reported discrepancy in predicted and detected molecular weights (Rohrer and Rawlings, 1992) corroborates the idea that the sequence shown here is correct.

It was of interest to note that the omega sequence DGRGG (Shurvington *et al.*, 1992) was well-conserved in the two proteins. However, no obvious candidate for an nuclear localization sequence NLS was seen in the MobA sequence.

In vitro *cleavage by VirD2*

Purified VirD2 was used to investigate its ability to cleave a synthetic oligonucleotide that contained the nick region of the pTF-FC2 plasmid or the pTiA6 right border sequence from the octopine

strain of *A. tumefaciens*. Cleavage products obtained following incubation of VirD2 with the 41-mer pTF-FC2 *oriT* oligonucleotide and the 35-mer pTiA6 right border oligonucleotide were separated in a denaturing polyacrylamide gel. In both cases, a main product with the electrophoretic mobility of a 13-mer and a slightly smaller band were observed (Figure 2). These results showed that VirD2 is able to recognize the *oriT* region and is able to cleave the synthetic oligonucleotide with an efficiency of 45% when compared with the efficiency of cleaving the right border (36% cleavage versus 80% cleavage). The occurrence of a 13-mer in the cleavage of the pTF-FC2 *oriT* gives the exact cleavage site of the *oriT* nick region (Figure 2, Table 2). The identification of this site had hitherto not been proven experimentally.

In vivo *transfer by MobA and VirD2*

Our previous results indicated pTF-FC2 MobA-mediated transformation occurred with a proficiency that was similar to VirD2-dependent T-DNA transfer (Dube and Thomson, 2003). In this study we tested the efficiency of DNA transfer

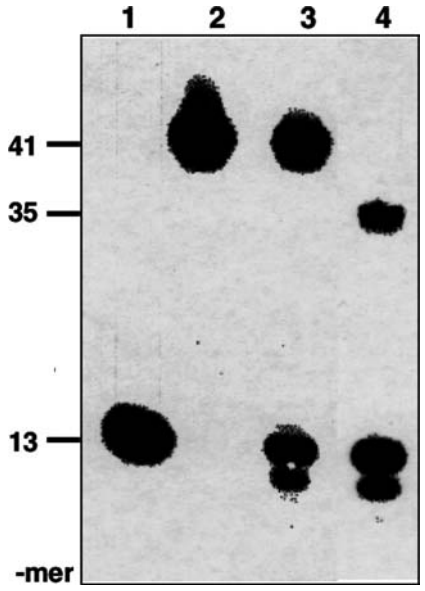

Figure 2. In vitro cleavage by purified VirD2 protein of oligonucleotides containing the pTF-FC2 origin of transfer region and the T-DNA border region, respectively. Lane 1, 13-mer marker; lane 2, 41-mer oligonucleotide; lane 3, 41-mer oligonucleotide-containing pTF-FC2 *oriT* reacted with VirD2; lane 4, 35-mer containing the tumor-inducing (Ti) plasmid border, reacted with VirD2.

Table 2. Alignment of nick regions of plasmids belonging to different incompatibility groups.

Group	Name of plasmid	Nick region	Nicking enzyme
Inc P/Q	pTF-FC2	GGTCATCCTG$^{\nabla}$TAT	MobA
Inc P	RK2/RP4	ACCTATCCTG$^{\nabla}$CCC	TraI
Inc P	pTi C58	ATATATCCTG$^{\nabla}$CCA	VirD2
Inc Q	RSF1010	GGTAAGTGCG$^{\nabla}$CCC	MobA

to young tobacco seedlings by the *Agrobacterium* strains shown in Table 1. Plasmid pDER-*bar*-GUS-Δ*Mob*A, containing an internal deletion of 1084 bp of its 1228 bp sequence was used as MobA defective pTF-FC2 plasmid (Figure 3). In the first assay, the activity of β-glucuronidase transiently expressed by the *uidA* gene carried on vectors pDER-*bar*-GUS and pDER-*bar*-GUS-Δ*Mob*A represents a quantitative measure of transferred DNA molecules which arrive in the plant cell nucleus without necessarily being integrated into the genome (transfer efficiency). Thus, transfer efficiency can be described as the number of blue spots appearing on a seedling after histochemical staining. Transient expression experiments showed nearly equal numbers of blue spots on cotyledons inoculated with GV3101 (pPM6000K)(pDER-*bar*-GUS) and GV3101 (pPM6000)(pDER-*bar*-GUS) (Table 3). This suggests that MobA is able to transfer T-DNA as efficiently as the combination of VirD2 and MobA. Inoculation of tobacco cotyledons with GV3101 (pPM6000K)(pDER-*bar*-GUS-Δ*Mob*A) showed no DNA transfer and transfection with an undiluted GV3101 (pPM6000) (pDER-*bar*-GUS-Δ*Mob*A) strain showed very

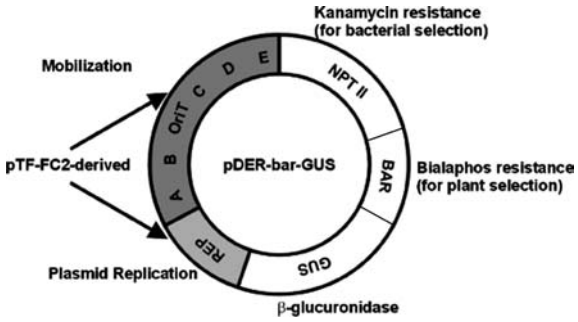

Figure 3. Simplified map of plasmid pDER-*bar*-GUS. A–E stand for *MobA*, *MobB, MobC, MobD,* and *MobE*. The sizes of the indicated genes are not to scale. pDER-*bar*-GUS-Δ*MobA* is identical except for a deletion in *MobA*. For a detailed description see Materials and methods.

Table 3. Efficiency of DNA transfer to plants and of stable transformation of plants.

	Relevant genotype of *Agrobacterium* strains		DNA transfer		Transformation		Integration (transformation/ DNA transfer)
	*Vir*D2	*Mob*A	Number of GUS spots/number of plants	Number of GUS spots/plant	Number of calli/number of plants	Number of calli/plant	
(a)	−	+	1550/298	5.2	192/1234	0.156	0.030
(a)	+	+	1561/332	4.7	145/942	0.154	0.033
(b)	+	−	2/350	6×10^{-5}	0/∼1000	$< 1 \times 10^{-5}$	NA
(b)	−	−	0/∼300	$< 3 \times 10^{-5}$	0/∼1000	$< 1 \times 10^{-5}$	NA

(a) Bacterial suspension was diluted 100-fold.
(b) Bacterial suspension was used undiluted; number of spots/calli was divided by 100, for comparison with data obtained using diluted bacterial suspensions.
NA, not applicaple.

little transfer; about 0.03% of the transfer efficiency of the 1:100 diluted strain of either GV3101 (pPM6000)(pDER-*bar*-GUS) or GV3l0l (pPM6000K)(pDER-*bar*-GUS) was detected. These surprising results show that although VirD2 is able to cleave the *oriT* region *in vitro* with a 40% efficiency, *in vivo* transformation frequencies are very low. There is a possibility that VirD2 is able to cleave the *oriT* region efficiently also *in vivo* but is unable to transfer this DNA to plant cells.

The efficiency of T-DNA integration into the plant chromosomal DNA was assayed after selection for phosphinothricin (PPT)-resistant calli (Table 3). PPT, an inhibitor of glutamine synthetase in both plants and bacteria, is a major constituent of bialaphos. The *bar* gene product is able to inactivate PPT by acetylating it (Thompson *et al.*, 1987). The relative transformation frequency of a strain corresponds to the number of PPT-resistant calli per seedling transformed compared with this number for a wild-type bacterium (Tinland *et al.*, 1995). The integration efficiency is defined as the ratio of transformation efficiency to transfer efficiency. This value represents the fraction of T-DNA molecules that integrate from a pool that entered the nucleus.

The experiment to test for stable integration of the plasmid DNA showed that both strains GV3101 (pPM6000)(pDER-*bar*-GUS) and GV3I01 (pPM6000K)(pDER-*bar*-GUS) transformed tobacco seedlings with a similar frequency of 15.4% and 15.6%, respectively. Comparison of T-DNA transfer to plants with the help of VirD2 and pTF-FC2 transfer to plants using its own mobilization functions showed an almost identical efficiency.

Discussion

VirD2 is one of the key *A. tumefaciens* proteins involved in T-DNA processing and transfer. It contains a strand transferase domain, necessary for nicking the T- DNA borders, a bipartite C-terminal NLS, necessary for piloting the T-strand to the plant nucleus, and a conserved region called ω that is important for virulence (Gelvin, 2000; Tzfira and Citovsky, 2002).

Cleavage by VirD2 of single-stranded oligonucleotides containing T-DNA border sequences has been demonstrated *in vitro* (Pansegrau *et al.*, 1993). Therefore, it was not totally surprising that purified VirD2 protein could cleave the origin of transfer region of pTF-FC2, since it is comparable, but not identical in sequence to the T-DNA border sequences (Figure 2). This once more demonstrates that VirD2 has a rather relaxed specificity; also the transfer origin of plasmid RP4 was cleaved by VirD2, although the RP4 relaxase TraI could cleave only its cognate substrate (Pansegrau *et al.*, 1993).

In view of these *in vitro* results the outcome of deletional analysis to determine the roles played by VirD2 or pTF-FC2 MobA in the transfer of DNA to plant cells was totally surprising. The efficient plant transformation results presented earlier could have been due to either activity of VirD2 or the pTF-FC2's mobilization region present in the

agrobacterial strain used. However, our present dissection of the system clearly showed that transfer as well as integration is entirely due to MobA. The other mobilization proteins also present on the plasmid may aid in the process but are inactive in the absence of MobA. A step in which VirD2-mediated pTF-FC2 transfer could be blocked could be the requirement for VirD1 in cleavage of one strand of the normally double-stranded substrate. This has been shown *in vitro* (Scheiffele *et al*., 1995) and *in vivo* (Filichkin and Gelvin, 1993) and is consistent with the finding that VirD1 and VirD2 are interacting (Relic *et al*., 1998). Other possibilities for incompatibilities between VirD2 and the plasmid transfer origin could be in the lack of covalent-binding of VirD2 to the 5′- terminus, a deficiency in export of the plasmid DNA/VirD2 complex and/or problems of this complex with entering the plant cell or the nucleus. Interestingly, the presence of VirD2 does not interfere with the activity of MobA. This finding is consistent and comparable with that made by Shadenkov *et al*. (1996) and Bravo-Angel *et al*. (1999) who both found that DNA transfer by MobA of RSF1010 is neither dependent on, nor inhibited by, the presence of VirD2.

It has been reported that RSF1010 MobA-mediated transformation occurs with a 100-fold lower efficiency compared with VirD2-dependent T-DNA transfer (Bravo-Angel *et al*., 1999). In contrast, pTF-FC2 MobA-mediated transformation occurred with an efficiency that was similar to VirD2-dependent T-DNA transfer. Inspection of the MobA sequence of pTF-FC2 only partially explains this. There is a good correlation of the three motifs generally found in relaxases; the invariant tyrosine (position 25 of MobA and 29 in VirD2), essential for covalent-binding of the relaxases to the 5′-termini of their cognate border/origin of transfer sequences, is present. However, there is no canonical NLS sequence; experimental results will have to replace *in silico* analysis. Interestingly, the MobA protein does contain an omega-like sequence. Definition of its importance will also require experimentation, especially in view of the fact that the relevance for T-DNA transfer and integration of this sequence in VirD2 remained controversial (Shurvington *et al*., 1992; Bravo-Angel *et al*., 1998: Gelvin, 2000).

For application as a useful, novel and efficient alternative to T-DNA-mediated transformation a number of possible improvements of pTF-FC2 MobA could be envisaged: its expression could be made inducible, possibly by plant phenolic compounds; a readily recognizable NLS could be implanted and the initiation codon GTG, as now involved in start of translation, could be changed to an ATG which will be more efficiently used.

Acknowledgements

Authors are grateful for the help by the collaborators of the Thomson and Hohn laboratories. Special thanks are due to Cynthia Ramos (Basel) for conducting plant work, to Erich Lanka for discussions, to Thomas Hohn for artwork, and to Olivier Fritsch for help in bioinformatics. TD was the recipient of a short-term UNESCO fellowship in biotechnology. We also acknowledge the financial help of the Novartis Research Foundation to Barbara Hohn.

References

Albright, L.M., Yanofsky, M.F., Leroux, B., Ma, D. and Nester, E.W. 1987. Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single stranded T-DNA. J. Bacteriol. 16: 1046–1055.

Bhattacharjee, M.K. and Meyer, R.J. 1991. A segment of a plasmid gene required for conjugal transfer encodes a site specific, single-strand DNA endonuclease and ligase. Nucleic Acids Res 19: 1129–1137.

Bonnard, G., Tinland, B., Paulus, F., Szegedi, E. and Otten, L. 1989. Nucleotide sequence, evolutionary origin and biological role of a rearranged cytokinin gene isolated from a wide host range biotype III *Agrobacterium* strain. Mol. Gen. Genet. 216: 428–438.

Bravo-Angel, A.M., Hohn, B. and Tinland, B. 1998. The omega sequence of VirD2 is important but not essential for efficient transfer of T-DNA by *Agrobacterium tumefaciens*. Mol. Plant Microbe-Interact. 11: 57–63.

Bravo-Angel, A.M., Gloeckler, V., Hohn, B. and Tinland, B. 1999. Bacterial conjugation protein MobA mediates integration of complex DNA structures into plant cells. J. Bacteriol. 181: 5758–5765.

Buchanan-Wollaston, V., Passiatore, J.E. and Cannon, F. 1987. The *mob* and oriT of a bacterial plasmid promote its transfer to plants. Nature 328: 172–175.

Cohen, A., Fisher, W.D., Curtis III, R. and Adler. H.I. 1986. DNA isolated from *Esherichia coli* mini cells mated with F^+ cells. Proc. Natl. Acad. Sci. USA 61: 61–68.

Dorrington, R., Bardien, S. and Rawlings, D. 1991. The broad-host-range plasmid PTF-FC2 requires a primase-like protein for autonomous replication in *Echerichia coli*. Gene 108: 7–14.

Dumas, F., Duckely, M., Pelzcar, P., van Gelder, P. and Hohn, B. 2001. An *Agrobacterium* VirE2 channel for

T-DNA transport into plant cells. Proc. Natl Acad. Sci. USA 98: 485–490.

Dürrenberger, F., Crameri, A., Hohn, B. and Koukolikova-Nicola, Z. 1989. Covalently bound VirD2 of *Agrobacterium tumefaciencs* protects the T-DNA from exonucleolytic degradation. Proc. Natl. Acad. Sci. USA 86: 9154–9158.

Filichkin, S.A. and Gelvin, S.B. 1993. Formation of a putative relaxation intermediate during T-DNA processing directed by the *Agrobacterium tumefaciens* VirD1, VirD2 endonuclease. Mol. Microbiol. 8: 915–926.

Gelvin, S.B. 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. Ann. Rev. Plant Physiol. Plant Mol. Biol. 51: 223–256.

Hansen, G. and Chilton, M.D. 1999. Lessons in gene transfer to plants by a gifted microbe. Curr. Topics Microbiol. Immunol. 240: 21–58.

Henderson, D. and Meyer, R. 1999. The MobA-linked primase is the only replication protein of R1162 required for conjugal mobilization. J. Bacteriol. 181: 2973–2978.

Howard, E.A., Winsor, B., De Vos, G. and Zambryski, P. 1989. Activation of the T-DNA transfer process in *Agrobacterium* results in the generation of a T-strand-protein complex: tight association of VirD2 with the 5′ ends of T-strands. Proc. Natl Acad. Sci. USA 86: 4017–4021.

Lanka, E. and Lessl, M. 1994. Common mechanisms in bacterial conjugation and Ti-mediated T-DNA transfer to plant cells. Cell 77: 321–324.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum 15: 473–497.

Pansegrau, W., Schoumacher, F., Hohn, B. and Lanka, E. 1993. Site specific cleavage and joining of single stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids; analogy to bacterial conjugation. Proc. Natl Acad. Sci. USA 90: 11538–11542.

Pansegrau, W., Schörder, W. and Lanka, E. 1994. Concerted action of three distinct domains in the DNA cleaving-joining reaction catalyzed by relaxase (TraI) of conjugative plasmid RP4. J. Biol. Chem. 269: 2782–2789.

Perwez, T. and Meyer, R. 1999. Stabilization of the relaxosome and stimulation of conjugal transfer are genetically distinct functions of the R1162 protein MobB. J. Bacteriol. 181: 2124–2134.

Puchta, H. and Hohn, B. 1991. The mechanism of extrachromosomal homologous DNA recombination in plant cells. Mol. Gen. Genet. 230: 1–7.

Rawlings, D.E., Pretorius, I. and Woods, D.R. 1984. Expression of a *Thiobacillus ferooxidans* origin of replication in *E. coli*. J. Bacteriol. 158: 737–738.

Rawlings, D.E. and Tietze, E. 2001. Comparative biology of IncQ and IncQ-like plasmids. Microbiol. Mol. Biol. Rev. 65: 481–496.

Ream, W. 1989. *Agrobacterium tumefaciens* and interkingdom genetic exchange. Ann. Rev. Phytopathol. 27: 583–618.

Relic, B., Andjelkovic, M., Rossi, L., Nagamine, Y. and Hohn, B. 1998. Interaction of the DNA modifying proteins VirD1 and VirD2 of *Agrobacterium tumefaciens*: analysis by subcellular localization in mammalian cells. Proc. Natl Acad. Sci. USA 95: 9105–9110.

Rohrer, J. and Rawlings, D. 1992. Sequence analysis and characterization of the mobilization region of a broad-host plasmid, pTF-FC2, isolated from *Thiobacillus ferooxidans*. J. Bacteriol. 174: 6230–6237.

Rossi, L., Hohn, B. and Tinland, B. 1993a. The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plants. Mol. Gen. Genet. 239: 345–353.

Rossi, L., Escudero, J., Hohn, B. and Tinland, B. 1993b. Efficient and sensitive assay for T-DNA-dependent transient gene expression. Plant Mol. Biol. Rep. 11: 220–229.

Sambrook, J. and Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Scheiffele, P., Pansengrau, W. and Lanka, E. 1995. Initiation of *Agrobacterium tumefaciens* T-DNA processing: purified protein VirD1, VirD2 catalyze site- and strand-specific cleavage of super helical T-border DNA *in vitro*. J. Biol. Chem. 270: 1269–1276.

Scherzinger, E., Bagdasarian, M.M., Scholz, P., Lurz, R., Ruckert, B. and Bagdasarian, M. 1984. Replication of the broad-host-range plasmid RSF1010: requirement for three plasmid-encoded proteins. Proc. Natl Acad. Sci. USA 81: 654–658.

Shadenkov, A.A., Kovaleva, M.V., Kuzmin, E.V., Uzbekova, S.V. and Shemyakin, M.F. 1996. VirD2-independent but MobA-dependent transfer of broad-host-range plasmid RSF1010 DNA from *Agrobacterium* into plant cell nucleus. Mol. Biol. 30: 272–275.

Shurvinton, C.E., Hodges, L. and Ream, W. 1992. A nuclear localization signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. Proc. Natl Acad. Sci. USA 89: 11837–11841.

Stachel, S.E. and Zambryski, P.C. 1986. *Agrobacterium tumefaciens* and the susceptible plant cell: a novel adaptation of extracellular recognition and DNA conjugation. Cell 47: 155–157.

Stachel, S.E., Timmerman, B. and Zambryski, P. 1986. Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. Nature 322: 706–711.

Thompson, C.J., Rao Movva, N., Tizard, R., Crameri, R., Davies, J.E., Lauwereys, M. and Botterman, J. 1987. Characterization of the herbicide-resistance gene bar from *Streptomyces hygroscopicus*. EMBO J. 6: 2519–2523.

Tinland, B., Schoumacher, F., Gloeckler, V., Bravo-Angel, A.M. and Hohn, B. 1995. The *Agrobacterium tumefaciens* virulence protein D2 is responsible for precise integration of T-DNA into the plant genome. EMBO J. 14: 3585–3595.

Tzfira, T. and Citovsky, V. 2002. Partners-in-infection: host proteins involved in the transformation of plant cells by *Agrobacterium*. Trends Cell Biol. 12: 121–129.

Wang, K., Stachel, E., Timmerman, B., Van Montagu, M. and Zambryski, P. 1987. Site specific nick in the T-DNA border sequence as a result of *Agrobacterium vir* gene expression. Science 235: 587–591.

Yanofsky, M.F., Porter, S.G., Young, C., Albright, L.A., Gordon, M.P. and Nester, E.W. 1986. The *virD* operon of *Agrobacterium* encodes a site-specific endonuclease. Cell 47: 471–477.

Zhang, S. and Meyer, R.J. 1997. The relaxosome protein MobC promotes conjugal plasmid mobilization by extending DNA strand separation to the nick site at the origin of transfer. Mol. Microbiol. 25: 509–516.