Genetic Analysis of the *virD* Operon of *Agrobacterium tumefaciens*: a Search for Functions Involved in Transport of T-DNA into the Plant Cell Nucleus and in T-DNA Integration

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The transferred DNA (T-DNA) is transported from *Agrobacterium tumefaciens* to the nucleus and is stably integrated into the genome of many plant species. It has been proposed that the VirD2 protein, tightly attached to the T-DNA, pilots the T-DNA into the plant cell nucleus and that it is involved in integration. Using agroinfection and β -glucuronidase expression as two different very sensitive transient assays for T-DNA transfer, together with assays for stable integration, we have shown that the C-terminal half of the VirD2 protein and the VirD3 protein are not involved in T-DNA integration. However, the bipartite nuclear localization signal, which is located within the C terminus of the VirD2 protein and which has previously been shown to be able to target a foreign protein into the plant cell nucleus, was shown to be required for efficient T-DNA transfer. *virD4* mutants were shown by agroinfection to be completely inactive in T-DNA transfer.

The soil bacterium *Agrobacterium tumefaciens* transfers a segment of its genetic information into plant cells. The transferred DNA (T-DNA) is stably integrated into the plant genome, where its expression leads to the synthesis of plant hormones and thus to the tumorous growth of the cells. The T-DNA, delimited by two almost perfect direct 25-bp repeats, is normally part of the large tumor-inducing (Ti) plasmid. However, the T-DNA is processed, i.e., liberated from the Ti plasmid, in response to signal compounds (such as acetosyringone) excreted by wounded plant cells. Proteins encoded by the virulence genes, which are located on the Ti plasmid and grouped into at least six operons (*virA*, *-B*, *-C*, *-D*, *-E*, and *-G*), mediate the actual transfer to the plant (for reviews, see references 28, 38, 58). Several of these proteins form a complex with the T-DNA and thus probably assist it on its journey: export from the bacterial cell, entry into the plant cell and the plant cell nucleus, and finally integration into the plant genome. The VirE2 and VirD2 proteins, encoded by the second open reading frame (ORF) of the *virE* and *virD* operons, respectively, have been shown to interact with free T-DNA molecules. VirE2 is a nonspecific DNA-binding protein (7, 9, 17) which attaches to single-stranded T-DNA, the T-strands (6). VirD2, together with the VirD1 protein, is involved in nicking at the 25-bp repeats, and it remains very tightly (most probably covalently) attached to the processed T-DNA (13, 20, 24, 53, 57). Only the N-terminal half of the VirD2 protein is required for the nicking reaction (10, 56), whereas the C-terminal half may be involved in later stages of transformation. The VirD2 protein contains two nuclear localization signals (NLS) at each end: the N-terminal sequence is of the single-cluster type, whereas the C-terminal sequence belongs to the bipartite NLS group (52) (see also Fig. 3A). Foreign proteins fused to either of the two NLS sequences have been shown to be targeted into the plant cell nucleus (21, 25, 48). Thus, the VirD2 protein attached to T-DNA may be responsible for

the transport of the T-DNA into the plant cell nucleus. Analysis of remnants of the 25-bp border sequences of T-DNA integrated into the plant genome suggests that a protein attached to the right end of the T-DNA, and thus most probably the VirD2 protein, is involved in integration (16, 32).

Bacterial proteins involved in a reaction pathway are very often encoded by genes grouped in the same operon. Apart from the genes coding for the VirD1 and VirD2 proteins, there are two other genes, *virD3* and *virD4*, located within the *virD* operon. The production of the VirD4 protein by acetosyringone-induced bacteria has been demonstrated previously (35), but the production of the VirD3 protein has not been investigated. While the *virD4* gene has been shown to be essential for tumor formation (37, 45), the *virD3* gene has not been studied. A fifth ORF (ORF5), which is not required for virulence, is located immediately downstream of the *virD4* gene (27, 37).

In this study we investigated whether proteins encoded by the *virD* operon are involved in T-DNA integration. We looked for *virD* mutants which cannot integrate T-DNA into the plant genome. Such mutants should transfer the T-DNA into the plant cell but should not be tumorigenic. We used two different assays to detect T-DNA transfer independent of integration: agroinfection and the transient expression of a T-DNA-located marker gene. In agroinfection, a multimer of a viral genome is inserted between the T-DNA border sequences (18). Upon transfer to the plant cell, the viral genome escapes from the T-DNA by recombination or by replication (i.e., by transcription and reverse transcription in the case of cauliflower mosaic virus [CaMV] [19]). The resulting virus then replicates and spreads within the plant, giving rise to viral symptoms (see Fig. 1A). This constitutes a very sensitive method for the detection of T-DNA transfer. The expression of *Escherichia coli* β -glucuronidase (GUS) in plants following T-DNA transfer was used as an independent, very sensitive, transient assay (4, 26, 49). Tumor formation and the formation of calli resistant to kanamycin were used as assays for T-DNA transfer followed by stable

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integration. Transposon mutants of all *virD* genes were first screened by agroinfection. Since transposon mutations are often polar over the downstream-located genes, we constructed and tested several nonpolar deletion mutants of the *virD2* and *virD3* genes. By precisely deleting only the bipartite C-terminal NLS of the VirD2 protein, we directly tested its activity in vivo.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. A348 (15) is *Agrobacterium tumefaciens* A136, which is a Ti plasmid-less derivative of C58 (54), containing the wild-type octopine Ti plasmid pTiA6. The mutant *A. tumefaciens* strains are A136 containing the respective mutant Ti plasmids (45). Plasmids pBG5 (42a) and pEAP42 (18), both containing a bacterial kanamycin resistance gene, were mobilized into the above strains by triparental mating (11) by using HB101(pRK2013) as the helper strain (14). These strains were maintained on YEB (18) agar supplemented with appropriate antibiotics (kanamycin, 50 µg/ml; rifampin, 5 µg/ml; and carbenicillin, 100 µg/ml). The following plasmids were used for *virD* constructions: pUC7 (50), containing an ampicillin resistance gene; pLT89 (12), which is a derivative of pUC18, containing a symmetric polylinker and ampicillin resistance gene; pARO181 (36), containing a kanamycin resistance gene; and pMY1153 (56), which contains the *virD* region of the octopine Ti plasmid pTiA6 under the control of the Tac promoter. The *E. coli* host JM101 (55) was used for cloning and was maintained on M9 (41) medium supplemented with the appropriate antibiotics (ampicillin, 50 µg/ml, and kanamycin, 50 µg/ml).

Sequence analysis of transposon insertions. The intermediate vectors used to construct the pTi304 and pTi307 plasmids (45) were analyzed by using oligonucleotide primers originating from the ends of the inserted transposon. A sequencing kit from U.S. Biochemical Corp. (Cleveland, Ohio) was used according to the supplier's recommendations.

Construction of mutant Ti plasmids. (i) *virD2* mutants. The *EcoRI* fragment of the octopine Ti plasmid containing the *virD1*, *virD2*, and *virD3* genes under the control of the Tac promoter was subcloned from the plasmid pMY1153 into the vector pLT89, resulting in pV63EcoD. An internal *SalI* fragment containing part of the *virD2* and *virD3* genes was also subcloned into pLT89. This plasmid was digested with *BamHI* and *KpnI*, and a short DNA fragment generated by annealing the partially complementary oligonucleotides 5'-GATCCCCGGGCTGCAGGAATTCGGTAC-3' and 5'-CGAATTCCTGCAGCCCCGGG-3' and thus containing *BamHI* and *KpnI* cohesive ends was ligated to it. This resulted in the insertion of the amino acids Pro-Gly-Leu-Gln-Glu-Phe into the VirD2 protein truncated by the *BamHI-KpnI* deletion. This new *SalI* fragment was reinserted into pV63EcoD, thereby replacing the wild-type *SalI* fragment. The truncated *EcoRI* fragment of the resulting plasmid, pV63EcoDBKΔ, was inserted into the unique *EcoRI* site of the vector pARO181. The resulting intermediate vector, after transformation into *E. coli* S17-1 (44), was mobilized by conjugation into *A. tumefaciens* A136 containing the plasmid pTi304 as described previously (44). The mutant pTiVirD2Δ1 was obtained by screening for double recombinants between the pTi304 and the intermediate vector. A similar approach was used to generate the mutant Ti plasmids pTiVirD2Δ2 and pTiVirD2Δ3. Plasmid pV63EcoDBKΔ was digested to completion with *BamHI* and partially with *NruI*. Short DNA fragments, generated by annealing partially complementary

oligonucleotides and thus containing *BamHI* cohesive and *NruI* blunt ends, were ligated to the digested pV63EcoDBKΔ (restoring both sites). For the construction of pTiVirD2Δ2, the oligonucleotides 5'-GATCCCCGGCCTGCAGGAGTTCGGT CG-3' and 5'-CGACCGAACTCCTGCAGGCCGGG-3' were used, resulting in the addition of amino acids Pro-Gly-Leu-Gln-Glu-Phe-Gly into the VirD2 protein truncated by the *BamHI-NruI* deletion. For the construction of pTiVirD2Δ3, the oligonucleotide 5'-GATCCTGCAGCCAAAGCGTCCGCGTGAC CGTCACGATGGAGAATTGGGTGGACGCAAACGTG CAAGAGGTAATCGTCG-3' and its complementary partner (4 nucleotides shorter at its 3' end, creating the *BamHI* cohesive end) were used, resulting in the addition of amino acids Leu-Gln-Pro-Lys-Arg-Pro-Arg-Asp-Arg-His-Asp-Gly-Glu-Leu-Gly-Gly-Arg-Lys-Arg-Ala-Arg-Gly-Asn-Arg (the bipartite C-terminal NLS is underlined) into the truncated VirD2 protein. The rest of the construction was carried out as for pTiVirD2Δ1. All three oligonucleotide insertions were confirmed by sequence analysis and the respective deletions of the Ti plasmid by Southern blot analysis.

(ii) *virD3* mutants. The *BamHI* fragment 18 (37) of pTiA6 was cloned into the *BamHI* sites of pUC7 to generate pKS5. Polymerase chain reaction (PCR) technology was used to amplify a fragment of pKS5 from nucleotide 2419 (5'-TCATGGGTTCGACGCCAGCCTTACAT-3') to the *SphI* site at position 2882 (5'-CGCTGCAGATAACCAGCATGCTGC A-3'), creating a *SalI* restriction site at position 2424, 169 amino acids into the VirD3 protein. The polymerase chain reaction product was digested with *SalI* and *SphI* and used to replace the *SalI*-to-*SphI* fragment of pKS5 corresponding to positions 2119 to 2882 of the *virD* sequence (37), resulting in pVirD3Δ103 (deleted for 103 amino acids of VirD3). The *nptII* promoter and gene were cloned from Tn5 cut with *SalI* and *HindIII*. The *HindIII* site was converted to a *SalI* site by using linkers, and the fragment was cloned into the *SalI* site of pVirD3Δ103 to generate pKS17. This deletes a total of 135 amino acids from the C terminus of the truncated VirD3 protein. The *virD3* deletion with the *nptII* insertion was crossed into the Ti plasmid by electroporating (3) pKS17 into A348, selecting for resistance to kanamycin, and screening for sensitivity to carbenicillin. The deletion was confirmed by Southern blot analysis.

Agroinfection and tumor formation. (i) **Agroinfection.** The binary vector pEAP42 containing a 1.4-mer of the CaMV genome between the T-DNA borders (18) was mobilized into each of the various strains. A fresh overnight culture was washed twice with 10 mM MgSO₄ and concentrated to an optical density at 600 nm (OD₆₀₀) of 2 (in some experiments, 20). Tenfold dilutions of this culture were made with 10 mM MgSO₄ up to a dilution of 10⁻⁶. Three- to four-week-old turnip plants (*Brassica campestris* var. *rapa*) were inoculated by rubbing carborundum and 50 µl of the respective dilution onto two leaves each. Six to eight plants were inoculated with each dilution. The plants were scored for CaMV symptoms 2 to 8 weeks later. Work with *Agrobacterium* strains containing the pEAP42 plasmid as well as plant inoculations was carried out in a BL3 containment.

(ii) **Tumor formation.** Overnight cultures were treated as described above but concentrated to an OD₆₀₀ of 40 (in some experiments, 400) to start the dilutions. Three- to four-week-old turnip plants were inoculated by being wounded at the crown with a toothpick and given 5 µl of the respective dilution. Six to eight plants were inoculated with each dilution. Tumor formation was scored 2 to 8 (sometimes 12) weeks later.

Transient GUS expression and kanamycin-resistant callus

formation. The *E. coli* GUS gene under the control of the 35S promoter, with its authentic start codon removed by site-directed mutagenesis, was fused in frame to the first 87 bp of the ORFV of CaMV (42). This gene was inserted between the T-DNA borders of the binary vector pBIN19 (2), resulting in the plasmid pBG5. This plasmid was mobilized into each of the various strains. A fresh overnight culture of the strains to be tested was washed twice and concentrated to an OD₆₀₀ of 2 (in some experiments, 20) in 10 mM MgSO₄ supplemented with acetosyringone (Aldrich-Chemie, Steinheim, Germany) at a concentration of 200 μ M. Tenfold dilutions of this culture were made with the same solution up to a dilution of 10⁻⁶ or 10⁻⁷. Tobacco leaf pieces (about 1 cm²) from 4- to 5-week-old axenically grown tobacco plants (*Nicotiana tabacum* cv. Petit Havana line SR1) were vacuum infiltrated (0.4 atm [ca. 40 kPa]) for 5 min with the respective dilution. Leaf pieces originating from the same leaf were infiltrated with the same dilution of the mutant strain and the wild-type strain, since a large variation between individual leaves was observed. After cocultivation for 3 days at 25°C on MS medium (34) supplemented with acetosyringone at 200 μ M, GUS activity was detected histochemically by using 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc; Biosynth AG, Staad, Switzerland). The leaf pieces were soaked in 0.052% X-Gluc in 100 mM NaH₂PO₄ (pH 7.0) in the presence of 0.1% sodium azide and vacuum infiltrated for 10 min, and the reactions were continued for 24 to 48 h at 37°C in the dark. The leaf pieces were bleached by incubation in 70% ethanol. At the same time, leaf pieces were placed onto MS medium containing kanamycin (50 μ g/ml) and supplemented with hormones (6-benzylaminopurine and α -naphthalene acetic acid at 1.0 and 0.1 mg/liter, respectively) and cefotaxime and vancomycin (both at 500 μ g/ml) and were scored for kanamycin-resistant callus formation 2 to 4 weeks later. Five and ten leaf pieces were usually assayed for GUS expression and kanamycin-resistant callus formation, respectively, for each strain and at each dilution.

Estimation of efficiency. Tenfold dilution series of cultures of the wild-type strain and of each mutant strain were inoculated in parallel in each experiment. For each strain, the highest dilution yielding a response (tumors, symptoms, blue spots, or kanamycin-resistant calli) was taken as a measure of the efficiency. The relative efficiency of each mutant strain was expressed as the factor by which its highest active dilution differed from the highest active dilution of the wild type. For example, a mutant strain is estimated to be 10⁶ times less efficient than the wild-type strain when it gives tumors only at the concentration of an OD₆₀₀ of 400, whereas the wild-type strain gives tumors up to the 10⁶ dilution of the same starting concentration. With efficiency of the wild-type strain set to 1, the efficiency of the mutant strain is thus 10⁻⁶.

Homology search. DNA sequences of the *virD* regions of the *A. tumefaciens* octopine A6 and nopaline C58 and *Agrobacterium rhizogenes* A4b Ti plasmids (22, 37, 40) were used for comparison of the respective VirD3 and VirD4 proteins by using the program of the Genetics Computer Group (University of Wisconsin, Madison).

RESULTS

Assays for T-DNA transfer and integration. Transfer of T-DNA into plant cells by transposon generated mutants (37) and nonpolar deletion mutants (this study) of the *virD* operon was tested by four different assay systems: agroin-

fection, tumor formation, transient expression of the GUS gene, and selection for kanamycin-resistant calli. Tumor formation and kanamycin-resistant callus formation require stable integration of the T-DNA into the plant genome. The agroinfection and GUS assay systems, on the other hand, are thought to detect T-DNA transfer independently of integration. It is assumed, but not proven, that the viral genome can escape from and that the GUS gene is expressed from the incoming nonintegrated T-DNA molecule. Since GUS expression and agroinfection require transcription or recombination of the T-DNA, both assays detect T-DNA transfer into the plant cell nucleus. Thus, when discussing results from these two assays, we use the term "T-DNA transfer" to designate transport of T-DNA from bacterium as far as into the plant cell nucleus, without integration.

A modified GUS gene, which is active in the plant cell but is not expressed in *A. tumefaciens* (42a) (see also Materials and Methods), was used to detect T-DNA transfer by histochemical staining (Fig. 1B). Selection for stable expression of the *nptII* gene, which confers kanamycin resistance to the transformed plant cells, was used as a measure of T-DNA transfer followed by integration. The two tests can be directly compared since the two markers are located on the same T-DNA and the same experimental setup is used (see Materials and Methods).

To estimate the efficiencies of the mutant strains in the different assays, we performed dilution experiments using the wild-type strain as an internal standard in each experiment (see Materials and Methods). The efficiency of each strain is given by a value relative to the wild-type strain, thus making it possible to compare the efficiencies of the mutant strains in the different assays.

Screening of *virD* transposon mutants by agroinfection. Eleven different avirulent mutants containing the transposon Tn3HoHo1 inserted into the individual genes of the *virD* operon of the octopine Ti plasmid pTiA6 (45) were tested for T-DNA transfer by agroinfection. The positions of the transposon insertions in the *virD* operon, a compilation of data from the literature characterizing the different mutants and the results of agroinfection and tumor formation experiments with turnips are shown in Fig. 2. Two strains containing the mutant Ti plasmids pTi304 and pTi307 were positive for agroinfection, although they have been reported to be nontumorigenic (45). However, in plants inoculated with the mutant strains, symptoms appeared 1 to 2 weeks later than in those inoculated with the wild-type strain. The same results were obtained when these two strains were tested for agroinfection of *Nicotiana benthaminiana* by using the beet curly top virus (2a).

Other strains were negative for agroinfection. The strains containing pTi306, pTi311, and pTi334 were expected to be defective in T-DNA transfer since the transposon insertions are situated in the *virD1* gene and the 5'-terminal part of the *virD2* gene, which were previously shown to be required for nicking at T-DNA borders (56).

All four *virD4* mutants tested (Fig. 2) were likewise negative for agroinfection, which shows that the *virD4* gene is absolutely required for T-DNA transfer. The two ORF5 mutants, pTi327 and pTi342, were positive in this test. This confirms previous findings (37, 45) that ORF5 is not required for tumor formation.

Characterization of transposon insertion mutants positive for agroinfection. The precise locations of the transposon insertions in plasmids pTi304 and pTi307, which were positive for agroinfection, were determined by sequence analysis. The insertion in pTi304 was mapped to the 3' end of the

FIG. 1. Transient assays. (A) Agroinfection. (Left) A turnip leaf showing symptoms of CaMV infection about 3 weeks after inoculation with the wild-type strain containing the pEAP42 plasmid. (Right) A healthy turnip leaf. (B) Transient GUS expression detected by histochemical staining. Tobacco leaf pieces infiltrated with strains containing the pBG5 plasmid: the wild-type strain A348 (a and b) and the Ti plasmid-less strain A136 (c). A bacterial concentration of OD_{600} of 2 was used (see Materials and Methods). Photographs were taken on a Wild M8 stereomicroscope at a magnification of $\times 8$ (a and c) and $\times 16$ (b). About one-eighth of a 1-cm^2 leaf piece is shown in panels a and c.

FIG. 2. Screening of *virD* mutants by agroinfection and tumor formation on turnip plants. A schematic representation of the *virD* region of the octopine pTiA6 plasmid is shown. Individual *virD* genes are designated by D1 to D4 and ORF5. The numbers refer to the transposon mutants described previously (45). The locations of the transposon insertions are indicated by arrows. A compilation of data from previous studies characterizing the different mutants is given: border nicking (46, 56); T-strand production (46); T-DNA–VirD2 complex (57); and tumor formation on *Kalanchoe* plants (45). Since it is not possible to compare quantitatively the results from different assays performed in different laboratories, we refer to them only qualitatively (using + and –). We tested the different mutant strains for tumor formation and agroinfection on turnip plants. Bacteria concentrated to an OD₆₀₀ of 20 and 400 were used as inocula for the agroinfection and tumor formation experiments, respectively (see Materials and Methods); in both cases, the plants were screened after 6 weeks. Results are given in the two rows at the bottom. Each strain was tested in three different agroinfection experiments. In plants inoculated with strains containing pTi327 and pTi342, symptoms appeared as fast as in those inoculated with the wild-type strain A348 (+++), whereas plants inoculated with the strains containing pTi304 and pTi307 showed symptoms 1 to 2 weeks later (+).

virD2 gene, at positions 1687 to 1688 of the published DNA sequence (56). This insertion results in a fusion protein between the VirD2 protein and *E. coli* β -galactosidase encoded by the transposon, which is proficient in border nicking and is attached to the T-DNA (46, 56, 57). This transposon insertion was demonstrated to be nonpolar by complementation analysis (45).

In pTi307, the transposon is inserted into the 3' end of the *virD3* gene at positions 484 to 485 of the published sequence (37). The polarity of this mutation will be discussed later. Nonpolar deletion mutants were constructed for further studies of the functions of the VirD2 and VirD3 proteins.

***virD2* mutants.** The VirD2 protein of the mutant pTi304 plasmid lacks its last 73 amino acids. Therefore, our finding that the pTi304 plasmid is positive for agroinfection but not for tumor formation can be interpreted to mean that the C-terminal part of the VirD2 protein is essential only for stable integration into the plant genome and not for the T-DNA transfer itself. On the other hand, it has been proposed that the bipartite NLS which is also located within the last 73 amino acids of the VirD2 protein is involved in the targeting of the T-DNA into the plant cell nucleus (25, 47, 48, 52). To assess the function(s) of the C terminus of the VirD2 protein, three different in-frame deletion mutants were constructed (Fig. 3A). In plasmid pTiVirD2 Δ 1, 111 amino acids of the C terminus were deleted, but the last 38 amino acids, including the C-terminal NLS, remain intact. The short oligonucleotide used to construct the deletion resulted in the insertion of six additional amino acids into the truncated VirD2 protein. In plasmid pTiVirD2 Δ 2, 141 amino acids, including the bipartite C-terminal NLS, were deleted, but the last eight amino acids are intact. An oligonucleotide similar to that used in pTiVirD2 Δ 1 was inserted, resulting in the addition of the same six amino acids plus one additional amino acid. The sequence chosen for the oligonucleotide avoided creation of a new peptide sequence resembling an

NLS. To assess the function of the C-terminal NLS alone, the mutant pTiVirD2 Δ 3, in which an oligonucleotide coding for the bipartite C-terminal NLS and four adjacent amino acids (plus two additional amino acids) was reinserted into the deletion of pTiVirD2 Δ 2, was constructed (see Materials and Methods for details). The newly constructed mutants and the pTi304 mutant were assessed for their efficiency in agroinfection, transient GUS expression, and stable transformation assays. The results are summarized in Fig. 3A.

About one-fourth of the VirD2 protein can be deleted from the C terminus without any apparent effects. pTiVirD2 Δ 1 was as efficient as the wild-type Ti plasmid in all three assays (Fig. 3A). Thus, that part of the VirD2 protein deleted in this mutant is not required for T-DNA transfer or for integration in turnip and tobacco plants. Moreover, it is not required for tumor formation on stems of sunflower, mustard, or pea plants or on *Kalanchoe* leaves and stems (data not shown).

Whereas plasmid pTiVirD2 Δ 2 was 10 to 100 times less efficient in all four assays, plasmid pTiVirD2 Δ 3 was as efficient as the wild-type Ti plasmid. Thus, deletion of the C-terminal bipartite NLS significantly lowers the efficiency of transfer. It was shown that an octopine VirD2 protein lacking the same part of the C terminus (but lacking, in addition, the last eight amino acids) as the VirD2 protein encoded by pTiVirD2 Δ 2 is fully proficient in nicking at T-DNA borders (56); we used the same restriction site (*Bam*HI) for our constructions as was used to generate the truncated protein in this previous study (Fig. 3A). Moreover, pTiVirD2 Δ 3 behaves like the wild-type Ti plasmid. We thus can rule out the possibility that the lower transfer efficiency of pTiVirD2 Δ 2 is due to lower efficiency of T-DNA processing. The finding that the efficiency of pTiVirD2 Δ 2 is reduced to the same degree in all four assay procedures is consistent with the hypothesis that the C-terminal NLS is involved in transport of the T-DNA into the plant cell nucleus rather than in T-DNA integration.

FIG. 3. Efficiency of *virD2* and *virD3* mutants in four assays. (A) *virD2* mutants. A schematic representation of the *virD2* mutants is shown on the left side. Open bar, nucleotide sequence coding for the VirD2 protein; closed bar, nucleotide sequence coding for the N-terminal and the C-terminal NLS; horizontal line, extent of the deletion of the *virD2* gene; wavy line, β -galactosidase coding sequence; asterisk, putative casein kinase II phosphorylation site. The sites of the restriction enzymes which were used to construct the deletions are *Bam*HI (B), *Kpn*I (K), and *Nru*I (N). The numbers give the start and the end of the coding sequence in nucleotides. The estimated efficiencies of the different mutants in agroinfection, tumor formation (Tumors), transient GUS expression (GUS), and kanamycin-resistant callus formation (KmR calli) tests are given on the right side. At least three different experiments were performed to assess the efficiency of each mutant in each test (see Materials and Methods for estimation of efficiency). The mutant strains were always tested in parallel to the wild-type strain, except for agroinfection, where the strain containing pTi304 was compared with the wild-type strain and the strains containing the pTiVirD2Δ2 and pTiVirD2Δ3 plasmids were compared with the strain containing the pTiVirD2Δ1 plasmid. In this case, the efficiency of the latter strain was set to 1. In each experiment, the respective negative control, i.e., the Ti plasmid-less strain, was tested at the same time and never gave a positive response. The relative efficiency of this strain can thus be given as $<10^{-6}$ in agroinfection, $<10^{-6}$ in tumor formation, $<10^{-7}$ in transient GUS expression, and $<10^{-7}$ in kanamycin-resistant callus formation. For unknown reasons, the results of the agroinfection test showed more fluctuation between different experiments than did those of the other tests. (B) *virD3* mutants. A schematic representation of the *virD3* mutants is shown on the left side. Open bars, nucleotide sequence coding for the VirD3 protein; dashed line, *npt*II gene; wavy line, Tn3HoHo1 transposon. The estimated efficiencies of the mutants in agroinfection, tumor formation (Tumors), transient GUS expression (GUS) and kanamycin-resistant callus formation (KmR calli) tests are given on the right. At least three different experiments were performed to assess the efficiency of each mutant in each test (see Materials and Methods). The mutant strains were always tested in parallel to the wild-type strain. Negative controls are the same as described for panel A.

As can be seen from Fig. 3A, the strain containing pTi304 occasionally gave rise to very small, octopine-producing tumors (data not shown) on turnips after prolonged periods (2 to 3 months) and repeatedly gave rise to kanamycin-resistant calli on tobacco leaf pieces, although at a very low efficiency. The efficiency of pTi304 was also very low when assayed for transient GUS expression. However, although it was less efficient than the wild type in the agroinfection assay, this strain was about 100 times more efficient in this assay than in the tumor formation assay. The reason for this difference is not known (see Discussion).

***virD3* mutants.** We constructed the pTiVirD3Δ mutant by deleting about half of the *virD3* gene (a total of 135 amino acids was deleted from the C terminus of the putative VirD3 protein consisting of 201 amino acids; see Materials and Methods and Fig. 3B) and replacing this region with the *npt*II gene, including its own promoter. This mutant was shown by complementation analysis to be nonpolar over the *virD4* gene (data not shown). The mutant plasmid was as efficient

in tumorigenesis as the wild-type Ti plasmid on turnip plants (Fig. 3B). This demonstrates that the C-terminal part of the VirD3 protein (about two-thirds of the protein) is not required for any step of T-DNA transfer and integration. Moreover, the mutant *virD3* strain was as efficient as the wild-type strain when tested for tumorigenesis on *Kalanchoe* leaves and the stems of tomato, pea, and sunflower plants; tumors appeared without delay and were similar in size to those caused by the wild-type strain (data not shown). Thus, this mutant strain does not seem to be affected in host range. Likewise, deletion of the *virD3* gene from the *A. tumefaciens* nopaline Ti plasmid pTiT37 and the *A. rhizogenes* Ri plasmid pRiA4 had no effect on virulence (data not shown).

Plasmid pTi307 was tested in all four assays and found to be very inefficient in tumor formation, kanamycin-resistant callus formation, and transient GUS expression. However, as in the case of pTi304, it was more efficient in agroinfection than in tumor formation (Fig. 3B).

DISCUSSION

We used four different assay procedures to characterize *virD* mutants: tumorigenesis, kanamycin-resistant callus formation, agroinfection, and GUS expression. Whereas the first two assays require stable T-DNA integration, the latter two assays detect T-DNA transfer into the plant cell nucleus, assuming that they represent transient activity of incoming T-DNA molecules. Thus, comparison of the two types of assays should enable the identification of mutants defective in T-DNA integration. We base our conclusions on the comparison of GUS expression with the kanamycin-resistant callus formation for the following reasons: (i) these two assays are directly comparable, as described in Results; (ii) the results of the GUS assay showed less fluctuation than those of the agroinfection assay (Fig. 3); (iii) transient synthesis of the GUS protein following T-DNA transfer was recently demonstrated, which strongly suggests that the GUS expression assay is indeed independent of integration (31). From this comparison, we conclude that none of the mutants tested is deficient in T-DNA integration.

The two transposon mutants pTi304 and pTi307 were found to be more active in the agroinfection assay than in the tumor formation assay but were equally efficient in the two other assays. Interpretation of these results is difficult, especially in view of the fact that the *virD2* and *virD3* deletion mutants are tumorigenic.

We have shown that the VirD2 protein can be truncated without any effect on tumorigenicity on several plant species, e.g., 111 amino acids can be deleted from the C-terminal half without affecting activity. This suggests that the N- and C-terminal domains are independent of each other. Comparison of the amino acid sequence of VirD2 proteins from an octopine and a nopaline *A. tumefaciens* strain and from an *A. rhizogenes* strain showed that the N-terminal half is very well conserved (90% homology) but that the C-terminal half is not so well conserved (26% homology) (52). However, several blocks of amino acids present in the C terminus were shown to be completely conserved between the three strains (52), suggesting that they are important for the function of the protein. Two such adjacent blocks of amino acids are Lys-Arg-Pro-Arg (amino acids 396 to 399) and Arg-Lys-Arg-Ala-Arg (amino acids 409 to 413). These oligopeptides constitute the bipartite C-terminal NLS shown to be able to target a foreign protein into the plant cell nucleus (25, 48). Another block comprising the amino acids Gly-Pro-Ser-Gly-Ala (amino acids 346 to 350) is predicted to form a β turn in the protein (5). This block is deleted in the virulent mutant pTiVirD2 Δ 1 and is thus not essential for tumorigenicity. The function of the conserved C terminus, a conserved block located at the very end of the protein (Asp-Gly-Arg-Gly-Gly; amino acids 419 to 423), was not addressed in this study.

The function of the bipartite C-terminal NLS in T-DNA transfer was directly tested by comparing two mutants (pTiVirD2 Δ 3 and pTiVirD2 Δ 2) which differ by the presence or absence of this sequence. The mutant plasmid pTiVirD2 Δ 2 lacking the NLS is tumorigenic, although it is 10 to 100 times less efficient in all tests than the corresponding positive controls (wild type and pTiVirD2 Δ 3). The finding that this mutant is less efficient than the wild type in the two transient expression assays supports the idea that the transport of the T-DNA into the plant cell nucleus is mediated by the nuclear localization signals of the attached VirD2 protein (21, 25, 47, 48, 52).

The fact that pTiVirD2 Δ 2 is not completely negative in

transfer indicates that the C-terminal NLS is not absolutely essential. The N-terminal NLS, able to target a reporter protein to the plant cell nucleus (21, 48), was recently suggested not to be involved in T-DNA transfer (40a, 42b). The functional deficiency of the mutant lacking the C-terminal NLS may thus be complemented by another virulence protein of the T-DNA-protein complex such as VirE2, which has recently been shown to contain active NLSs (8). Moreover, a certain proportion of the T-DNA molecules may come into contact with the plant chromatin when the nucleus disintegrates, i.e., during cell division.

Putative casein kinase II phosphorylation sites, located 20 to 25 amino acids upstream of the C-terminal NLS of the VirD2 protein, have been found in different *Agrobacterium* strains (25). In the simian virus 40 large-T antigen such a site, while not essential, has been shown to be important for rapid accumulation of the protein in the nucleus (39). On the other hand, nuclear import of the *Saccharomyces cerevisiae* transcription factor SW15 is regulated by phosphorylation at such sites (33). However, the putative phosphorylation site was deleted in the mutant Ti plasmids pTiVirD2 Δ 1 and pTiVirD2 Δ 3 and thus is not required for T-DNA transfer (Fig. 3A).

The analysis of T-DNA integrated into plant genomes suggested that a protein attached to the right end of the T-DNA, and thus most probably the VirD2 protein, is involved in integration (16, 32). However, we did not find any *virD2* mutant that was proficient in transfer but defective in integration. Thus, the N-terminal half required for nicking at the border sequences may also be involved in integration. The reactive site involved in linking the protein to the T-DNA may also be involved in ligating the T-DNA to the target DNA, resembling the action of DNA topoisomerases (29). Moreover, similar to the activities of the bacteriophage ϕ X174 protein A (30), the attached VirD2 protein may even be involved in nicking of the target site. Such a mechanism may not allow the separation of the nicking and integrase activities by genetic means.

The very low overall efficiency of GUS expression and kanamycin-resistant callus formation mediated by plasmid pTi304 (Fig. 3B) could be due to a combination of several factors. Firstly, the VirD2 protein is fused to *E. coli* β -galactosidase, and this may significantly influence the efficiency of T-DNA transfer since β -galactosidase is known to inhibit translocation of fusion proteins across the bacterial membrane (43). Secondly, this fusion protein lacks not only the C-terminal NLS but also the conserved C terminus. At present, we cannot exclude the possibility that the latter is required for T-DNA transfer and/or integration.

The octopine *virD3* gene can be truncated to a significant degree without affecting tumorigenicity on several plant species, as was also recently reported (51). The same is true for the corresponding *A. tumefaciens* nopaline and *A. rhizogenes* *virD3* genes (data not shown) (23). The complete gene is thus not essential for any step of T-DNA transfer and integration, at least on the host plants tested. However, its function in the natural infection process may not be detectable in laboratory experiments. *A. tumefaciens* nopaline and *A. rhizogenes* A4b VirD3 proteins are very similar in size and sequence (673 and 678 amino acids, respectively, with about 66% homology), whereas the octopine protein is much smaller (201 amino acids). The N-terminal (amino acids 1 to 85) and C-terminal (amino acids 86 to 161) regions of the octopine VirD3 protein share about 39 and 30% amino acid homology with the N terminus (amino acids 1 to 85) and C terminus (amino acids 574 to 649), respectively, of its

nopaline counterpart. The nopaline VirD3 protein contains 488 additional amino acids inserted between these two blocks of homology. This suggests that the nopaline *virD3* gene contains an insertion element that possibly inactivates the gene. Since production of the VirD3 protein in *A. tumefaciens* has never been demonstrated, the possibility remains that the *virD3* gene is completely dispensable and may be an inactive remnant of evolution. Complementation experiments (data not shown) indicate that the low efficiency of the pTi307 plasmid in all assays (Fig. 3B) is a result of partial polarity of the transposon insertion over the downstream-located *virD4* gene.

All tested transposon mutants of the *virD4* gene are completely negative in T-DNA transfer, as supported by a recent report on transient GUS expression (31). This indicates that this gene is absolutely required for a step of T-DNA transfer from the bacterium into the plant cell nucleus. The octopine VirD4 protein shows a high degree of homology (81% homology) to its nopaline counterpart. It also shares extended amino acid sequence similarity with the TraG protein of the RP4 and R751 plasmids (59), which is involved in the conjugal transfer of these plasmids. Furthermore, the VirD4 protein was shown to be localized in the inner bacterial membrane (35) and was recently proposed to be part of the complex transporting the T-DNA across the bacterial membrane (1). Thus, this suggests that this protein functions within the bacterial cell and is consistent with our finding that the VirD4 protein is essential for T-DNA transfer.

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