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Agrobacterium T-strand production *in vitro*: Sequence-specific cleavage and 5′ protection of single-stranded DNA templates by purified VirD2 protein

(T-DNA/conjugational DNA transfer/relaxosome/Ti plasmid-encoded virulence proteins/plant transformation)

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ABSTRACT **Virulence proteins VirD1 and VirD2 are subunits of a relaxosome-like protein complex that mediates conjugational transfer of a Ti plasmid segment, the T-DNA, from *Agrobacterium* into higher plants. The VirD1–VirD2 complex binds to 25-bp repeats at the borders of the T-DNA and catalyzes sequence-specific nicking of the conjugative DNA strand (the T-strand) at the third base of these repeats. Nuclear localization signals present in VirD2 target the T-strand to plant cell nuclei. In addition, VirD2 probably plays a role in the high-frequency integration of the T-DNA into the plant genome by illegitimate recombination. Whereas *Agrobacterium* transformation of dicots is very efficient, T-DNA integration in most monocots can barely be detected. To develop an artificial T-DNA delivery system for monocots, a technique for efficient *in vitro* production of T-strand DNAs was established by using VirD1 and VirD2 proteins purified from overexpressing *Escherichia coli* strains. The topoisomerase-like VirD2 enzyme was shown to mediate precise, sequence-specific cleavage of T-DNA border sequences carried by single-stranded DNA templates, even in the absence of VirD1 protein. During this reaction, VirD2 remains covalently bound to the 5′ end of artificial T-strand DNAs. In contrast, VirD2, alone or in complex with VirD1, fails to nick linear double-stranded DNA templates *in vitro*.**

Virulence genes of Ti and Ri plasmids code for a specialized conjugation system that mediates the mobilization and transfer of a DNA segment, termed T-DNA, from *Agrobacterium* into plants. This system for gene transfer from prokaryotes to eukaryotes is activated when agrobacteria infect wounded plants and is controlled by plant-specific phenolics via a Ti plasmid-encoded sensory–regulatory system including VirA and VirG functions (1–3). Boundaries of the T-DNA are flanked by 25-bp imperfect direct repeats that share homology with conserved sequences of conjugational transfer origins of wide-host-range bacterial plasmids (4, 5).

Following induction of virulence (*vir*) gene expression by plant phenolics, the T-DNA borders are specifically processed by a protein complex carrying VirD1 and VirD2 protein subunits (6, 7). Studies of the interaction of T-DNA borders with VirD1 and VirD2 proteins in either *Agrobacterium* or *Escherichia coli* revealed that VirD1 is required for efficient binding of VirD2 to the border repeats. The VirD1–VirD2 complex is known as a sequence-specific endonuclease–topoisomerase that introduces a nick in the conjugative T-strand (the "bottom strand") at the third base of the 25-bp border (8–12).

Properties of the VirD1–VirD2 complex show significant similarity to relaxosomal proteins of conjugative bacterial plasmids (1, 5, 7, 13–20). After the nicking reaction, VirD2 remains associated with the 5′ end of the conjugative DNA strand (17–19). A unique feature of *Agrobacterium* T-DNA transfer is that, after the initiation of strand-replacement DNA synthesis from a nick at the right T-DNA border, the released T-strand becomes fully protected against nucleases by binding to a single-stranded-DNA-binding protein, VirE2, and transferred to plants through membrane pores formed by VirB proteins. Nuclear localization signals within the VirE2 and the pilot VirD2 protein lead the T-strand to plant cell nuclei (1, 3, 7). The T-DNA integrates into potentially transcribed chromosomal domains by illegitimate recombination. It has been suggested that the integration process is potentiated by the VirD2 protein, which may mediate the ligation of the T-strand to free 3′ ends provided by nicks or gaps within the plant DNA (21, 22). In this respect it is striking that whereas T-DNA integration is highly efficient in dicotyledonous plants, *Agrobacterium*-mediated transformation of monocots, if it occurs at all, leads to defective, highly rearranged, or amplified T-DNA inserts (1–3). Agrobacteria must provide for two different steps in plant transformations, (*i*) DNA delivery and (*ii*) DNA integration into the host genome. If the first step is limiting the efficiency of transformation of some monocots, the production of an artificial T-strand *in vitro*, to be delivered by DNA-coated microprojectiles, for example, should be of considerable use. Here we describe an efficient method for the production of VirD2-associated T-strand DNAs *in vitro*.

MATERIALS AND METHODS

Overproduction of VirD1 and VirD2 Proteins in *E. coli*. Open reading frames of genes *virD1* and *virD2* (23) were amplified by the polymerase chain reaction (PCR) using the Ti-plasmid C58 subclone pGV0361 (24) as template, *Taq* DNA polymerase (Perkin–Elmer), and oligodeoxynucleotide primers 5′-CGCGGATCCATATGTCGCAAGGCAGTAGGCC-3′ and 5′-ATGGATCCCTACAAGGCGTCTTTCAGCAG-3′ for *virD1* and 5′-CGCGGATCCATATGCCCGATCGAGCTCAAG-3′ and 5′-ATGGATCCCTATCTCCTATTTCCCCCACG-3′ for *virD2*. To avoid generation of translational gene fusions during cloning of the PCR products in expression vector pET3b (25), the 5′-end-specific PCR primers were designed so that they contained both *Nde* I and *Bam*HI sites, whereas the 3′-end primers included only a *Bam*HI site. The PCR products were subcloned into pET3b between the *Nde* I and *Bam*HI sites, and the nucleotide sequence of *vir* genes in plasmids pETvirD1 and pETvirD2 was verified by DNA sequencing using T7 DNA polymerase (26). *E. coli* BL21(DE3) cultures harboring pETvirD1 or pETvirD2 grown to OD_{600} of 0.5 at 37°C in NZCYM medium

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Abbreviation: IPTG, isopropyl β-D-thiogalactopyranoside.

(27) containing ampicillin (100 μg/ml) were induced by 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) to derepress *vir* gene transcription (25) and grown for an additional 3 hr before the cells were harvested by centrifugation.

Purification of VirD1 and VirD2 Proteins. Bacterial pellets were suspended in 50 mM Tris·HCl (pH 8.0), sonicated, and processed (28) to purify inclusion bodies. Denaturation of both VirD1 and VirD2 was performed at 37°C (29). After denaturation in the presence of 100 mM dithiothreitol, VirD2 was renatured overnight at 20°C, whereas VirD1 was renatured in the presence of 5 mM/0.5 mM (oxidized/reduced) glutathione at 10°C for 3 days. After overnight dialysis in buffer (20 mM Tris·HCl, pH 7.6/50 μM EDTA) at 4°C, both VirD1 and VirD2 were further purified by heparin-Sepharose column chromatography in 20 mM Tris·HCl, pH 7.6/50 μM EDTA buffer by eluting VirD1 with 250 mM NaCl and VirD2 with 380 mM NaCl. N termini of purified VirD1 and VirD2 were sequenced with an Applied Biosystems 477A protein sequencer.

Preparation of DNA Templates and Assays of VirD1 and VirD2 Activities. Oligonucleotides corresponding to the top strand and the bottom strand, or T-strand (see ref. 1 for nomenclature), of the right T-DNA border region of Ti plasmid C58 (Fig. 2*A*) were purified from 12% polyacrylamide/urea gels and labeled at their 5′ ends by T7 polynucleotide kinase and [γ-^{32}P]ATP (5000 Ci/mmol; 1 Ci = 37 GBq) as described (27). A longer T-DNA border fragment was subcloned from pPCV6NF (30) into the *Bam*HI–*Sph* I sites of plasmids pUC118 and pUC119, yielding constructs pRB118 and pRB119. For labeling of the 3′ end of the bottom strand, the right T-DNA border fragment was excised from pRB118 by *Pst* I and *Bam*HI, and the *Bam*HI site was filled in by using [α-^{32}P]dGTP (3000 Ci/mmol) and *E. coli* DNA polymerase Klenow fragment (27). For labeling of the 5′ end of the bottom strand, plasmid pRB118 DNA was cleaved by *Xba* I, dephosphorylated by calf intestinal phosphatase, labeled with [γ-^{32}P]ATP and T7 polynucleotide kinase, and digested further by *Bam*HI. Labeled double-stranded DNA probes were isolated from nondenaturing 8% polyacrylamide gels or denatured to yield single-stranded substrate DNAs purified by electrophoresis in 6% polyacrylamide/urea gels. VirD1 and VirD2 were incubated with labeled substrate DNAs in 40 μl of nicking-assay buffer (20 mM Tris·HCl, pH 9.0/5 mM $MgCl_2$/1 mM dithiothreitol/100 μM EDTA with bovine serum albumin at 100 μg/ml) for 1 hr at 37°C. After the reactions were stopped with 1% SDS, the DNA samples were extracted with phenol/chloroform, precipitated with ethanol, dissolved in 5 μl of formamide loading buffer, denatured for 3 min at 95°C, and loaded on sequencing gels (27). To assay for linkage of VirD2 to the 5′-end of nicked T-strand substrates, nicking reactions were performed in 20 μl of assay buffer for 1 hr at 37°C and terminated with Laemmli protein sample buffer (31). The samples were denatured at 95°C for 3 min, the protein–DNA complexes were resolved by SDS/10% PAGE, and the gels were dried and subjected to autoradiography.

RESULTS

Cloning and Overexpression of *virD1* and *virD2* Genes in *E. coli*. Coding regions of *virD1* and *virD2* (23) were amplified by PCR using plasmid pGV0361 DNA (24) as template and were cloned in pET3b to yield expression vectors pETvirD1 and pETvirD2. To produce VirD1 and VirD2 proteins in large quantities, plasmids pETvirD1 and pETvirD2 were transformed into *E. coli* BL21(DE3) (25). The levels of VirD1 and VirD2 were monitored during the time course of IPTG induction by SDS/PAGE (Fig. 1) in comparison to cells carrying the pET3b vector alone.

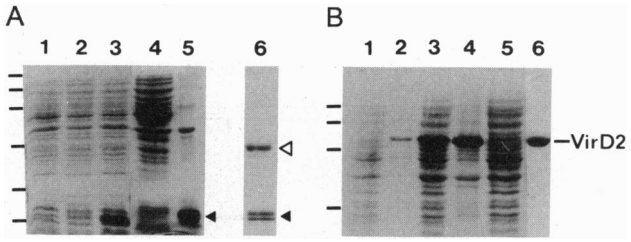

FIG. 1. Overexpression of *virD1* and *virD2* genes in *E. coli* and purification of their gene products. Quantities of VirD1 and VirD2 proteins were monitored in cell extracts before and after IPTG induction, as well as in the supernatant and inclusion-body fractions after sonication and in fractions obtained by heparin-Sepharose chromatography of renatured proteins. Bars to the left of *A* and *B* show the positions of molecular mass standards: from top to bottom, phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). (The last two standards are missing in *B*.) (*A*) Purification of VirD1. Lane 1, *E. coli* BL21(DE3) cells harboring pET3b, 3 hr after IPTG induction; lanes 2 and 3, BL21(DE3) cells carrying pETvirD1, before and 3 hr after IPTG induction, respectively; lanes 4 and 5, supernatant and inclusion-body fractions obtained after sonication and centrifugation, respectively; lane 6, 5 μg of heparin-Sepharose-purified VirD1. Open triangle, VirD1 dimer; filled triangle, VirD1 monomer. (*B*) Purification of VirD2. Lane 1, as in *A*; lanes 2 and 3, BL21(DE3) cells harboring pETvirD2, before and 3 hr after IPTG induction; lanes 4 and 5, inclusion-body and supernatant fractions after sonication, respectively; lane 6, 4 μg of VirD2 purified by heparin-Sepharose chromatography.

Purification and Characterization of VirD1 and VirD2. When expressed from the T7 promoter in *E. coli* (25), VirD1 and VirD2 accumulated in inclusion bodies (Fig. 1, lanes 4 and 5). To purify VirD1 and VirD2, inclusion bodies were solubilized in 6 M guanidinium hydrochloride and diluted 1:100-fold in a buffer containing arginine to facilitate renaturation and correct folding of proteins (29). After dialysis, the crude VirD1 and VirD2 fractions were purified by heparin-Sepharose chromatography to near homogeneity (Fig. 1, lanes 6). VirD1 was renatured in the presence of glutathione to promote the formation of disulfide bridges between the two cysteine residues present in VirD1. As indicated also by immunoblot analysis (data not shown), the renaturation promoted a partial dimerization of VirD1 (Fig. 1*A*, lane 6, upper band), that could be abolished by dithiothreitol treatment. VirD1 monomers appeared as a double band after SDS/PAGE (Fig. 1*A*, lane 6, lower bands). N-terminal sequencing of proteins eluted from this double band yielded a common VirD1 N terminus that was identical with the amino acid sequence deduced from the *virD1* DNA sequence (23). Because VirD1 prepared from inclusion bodies gave rise to only a single protein band of 16 kDa on SDS/PAGE, it is probable that the double band obtained after glutathione treatment of the protein corresponds to partially denatured isomers of VirD1. The apparent molecular mass by SDS/PAGE agrees well with a value of 16,130 Da calculated for VirD1 on the basis of the DNA sequence. In contrast, the homogeneous VirD2 protein migrated as a single band of 56 kDa on SDS/PAGE as described (8), although a molecular mass of 49,579 Da was calculated on the basis of the known DNA sequence (23).

Templates for *in Vitro* Nicking Assays with Purified VirD1 and VirD2. To determine the optimal conditions for T-strand production *in vitro* with purified VirD1 and VirD2, several DNA substrates were designed. Two oligonucleotides representing the top and bottom strand of the 25-bp T-DNA border repeat of pTiC58 (Fig. 2*A* and top line of *B*) were synthesized as single-stranded substrates. A double-stranded substrate was prepared using a longer T-DNA border frag-

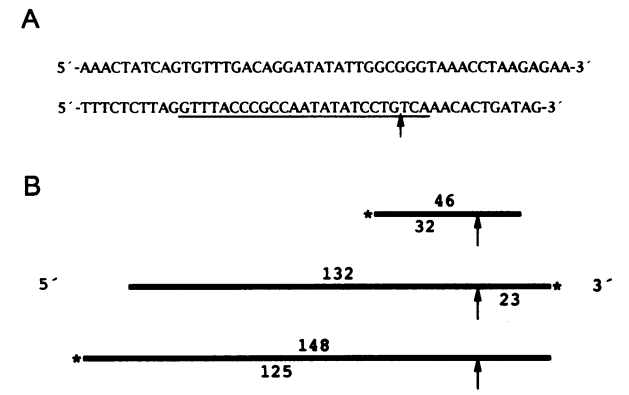

FIG. 2. DNA templates for nicking assays with VirD1 and VirD2. (*A*) Nucleotide sequence of single-stranded oligonucleotide templates representing the top strand and the bottom strand of the 25-bp border sequence (underlined) of the T-DNA of Ti plasmid C58. (*B*) Schematic of templates used in nicking assays. Top line, a 46-base single-stranded oligonucleotide corresponding to the bottom strand of the 25-bp T-DNA border sequence, labeled at its 5′ end by T7 polynucleotide kinase; middle line, bottom strand of the T-DNA border fragment (single-stranded substrate of 132 bases) labeled by DNA polymerase Klenow fragment at the 3′ end; bottom line, bottom strand of the T-DNA border fragment (single-stranded substrate of 148 bases) labeled at the 5′ end by T7 polynucleotide kinase. Stars indicate radioactively labeled DNA ends, arrows mark the position of VirD2 cleavage, and numbers below the bars indicate the size of cleavage products detected in nicking assays.

ment from plasmid pRB118. In addition, the bottom strand of this T-DNA border fragment (Fig. 2*B*, middle and bottom lines) was also used as single-stranded substrate. Both single-stranded and double-stranded DNA substrates were labeled at their 5′ ends by T7 polynucleotide kinase. To obtain selective labeling of the bottom strand, the double-stranded T-DNA border fragment was further digested by *Xba* I (Fig. 2*B*, bottom line, template of 148 bases). To assay for linkage between the 5′ end of the T-strand and VirD2, the T-DNA border fragment was labeled at the 3′ end of the bottom strand by DNA polymerase Klenow fragment and purified as a single-stranded template from sequencing gels (Fig. 2*B*, middle line, template of 132 bases).

VirD2 Catalyzes Sequence-Specific Cleavage of Single-Stranded DNA Templates. Sequence-specific nicking by VirD1, VirD2, or the VirD1–VirD2 complex was monitored by assays with single-stranded oligonucleotides representing the top or the bottom strand of the 25-bp T-DNA border repeat, using sequencing gels. Nicking assays performed with these single-stranded templates revealed that VirD2 exclusively uses the bottom strand of the T-DNA border sequence as substrate (Fig. 3*A*, lanes 3–8 and 9–14). Cleavage of the 5′-labeled bottom-strand oligonucleotide of 46 bases with increasing amounts of VirD2 correlated with the accumulation of a product of 32 bases. Densitometric evaluation of autoradiographs of different nicking assays indicated that on average about 50% of input substrate was processed by VirD2. This equilibrium reached by the nicking reaction was not changed by increasing the concentration of VirD2 or by prolonging the incubation period. At lower VirD2 concentrations, the amount of nicking products was proportional to the amount of VirD2 employed.

To determine the exact position of the VirD2 cleavage site, a Maxam–Gilbert G reaction (32) was performed with the bottom-strand template, and along with 5′-labeled single-stranded oligonucleotides of known size, the products of this reaction were run in parallel with a nicking assay in a sequencing gel. The result, shown in Fig. 3*A*, indicated that VirD2 cleavage *in vitro* occurred at the third guanine from the 3′ end, between the third and fourth base of the 25-bp T-DNA

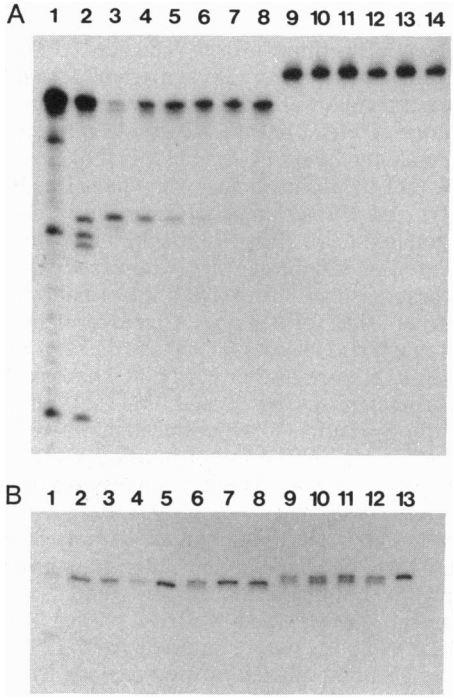

FIG. 3. *In vitro* sequence-specific cleavage of single-stranded DNA substrates by VirD2. (*A*) The 5′-end-labeled oligonucleotides representing either the bottom (lanes 3–8) or the top (lanes 9–14) strand of the T-DNA 25-bp border were incubated with various amounts of purified VirD2, electrophoresed in a 12% polyacrylamide/urea gel, and visualized by autoradiography. Lanes: 1, Maxam–Gilbert G reaction with the bottom strand (the difference in mobility of these fragments in comparison to lanes 2–7 is due to 3′-phosphorylated ends of the chemical cleavage products); 2, labeled size-marker oligonucleotides of 32, 30, 29, and 17 bases; 3–8, 0.3 ng (12 fmol) of bottom-strand oligonucleotide; 9–14, 0.3 ng of top-strand oligonucleotide. The amounts of VirD2 added to the samples were 3.8 μg (lanes 3 and 9), 1.9 μg (lanes 4 and 10), 0.95 μg (lanes 5 and 11), 0.48 μg (lanes 6 and 12), and 0.24 μg (lanes 7 and 13). VirD2 was omitted from samples loaded in lanes 8 and 14. (*B*) Analysis of the activity of VirD1 and VirD2 using a double-stranded DNA substrate. A double-stranded T-DNA border fragment isolated from plasmid pRB118 was selectively labeled at the 5′ end of the bottom strand and incubated with VirD1 (lanes 3, 4, 7, and 8), VirD2 (lanes 1, 2, and 6), or both VirD1 and VirD2 (lanes 9–12), and electrophoresed in an 8% polyacrylamide/urea gel before autoradiography. The double-stranded T-DNA border fragment was denatured before the addition of Vir proteins in samples 1–5. Each sample contained 0.8 ng (10 fmol) of DNA substrate. The samples contained 3.8 μg (lane 1) and 1.0 μg (lane 2) of VirD2, 1.15 μg (lane 3) and 4.6 μg (lane 4) of VirD1, no Vir protein (lane 5), 3.8 μg of VirD2 (lane 6), 1.15 μg (lane 7) and 4.6 μg (lane 8) of VirD1, and 3.8 μg of VirD2 in combination with 0.58 μg (lane 9), 1.15 μg (lane 10), 2.30 μg (lane 11), and 4.6 μg (lane 12) of VirD1 (corresponding to VirD1/VirD2 molar ratios of 1:1, 2:1, 4:1, and 8:1). Proteins were omitted from the sample in lane 13.

border sequence, as was previously determined for nicking by the VirD1–VirD2 complex *in vivo* (19).

VirD1 and VirD2 Are Not Sufficient for Nicking of Double-Stranded DNA Templates. Nicking assays performed with VirD2 and a 5′-end-labeled T-DNA border fragment indicated that VirD2 alone is not active on double-stranded DNA template (Fig. 3*B*, lane 6). To detect VirD2 cleavage, denaturation of the double-stranded template was necessary before incubation with VirD2 (Fig. 3*B*, lanes 1 and 2). In contrast, incubation of VirD1 with single- or double-stranded DNA templates did not reveal any DNA-modifying activity (Fig. 3*B*, lanes 3 and 4 and lanes 7 and 8). To test whether VirD1 would potentiate VirD2 activity on a double-stranded substrate or whether it would increase the efficiency of

VirD2-mediated cleavage of single-stranded DNA, both VirD1 and VirD2 were added to T-DNA border templates at various concentrations. These experiments indicated that VirD1 and VirD2 were not sufficient for cleavage of the 25-bp T-DNA border in double-stranded templates (Fig. 3*B*, lanes 9–12) and that VirD1 had no effect on VirD2 nicking activity on single-stranded DNAs (data not shown).

VirD2 Remains Associated with the 5′ End of the T-Strand After Cleavage of the 25-bp T-DNA Border Sequence *in Vitro*. Studies of the T-DNA border processing *in vivo* indicated that VirD2 remained stably, perhaps covalently, linked to the 5′ end of the T-strand (17–19). It was not known, however, whether this binding was mediated by the VirD2 protein or was the result of an interaction of VirD2 with other proteins involved in the process of T-DNA border cleavage. To test whether VirD2 would remain linked to artificial T-strand templates after the nicking reaction, the 3′-end-labeled bottom strand of a T-DNA border fragment (Fig. 2, template of 132 bases) was incubated with VirD2 and loaded without protease treatment onto a protein-denaturing polyacrylamide gel. Samples incubated with VirD2 resulted in a band shift on SDS/PAGE (Fig. 4*A*, lanes 1–4). The intensity of the slowly migrating labeled DNA band was proportional to the amount of VirD2 added to the samples. The mobility difference between this retarded DNA band and the VirD2 protein, visualized by Coomassie blue staining on the same gel, indicated a difference in molecular mass of about 8 kDa, corresponding to 23 nucleotides. (This estimate agreed well with the results obtained from nicking assays in which a bottom-strand substrate of 148 bases was 5′-labeled and cleaved by VirD2, resulting in a nicking product of 125 bases, 23 bases shorter than the substrate.) The retarded DNA band disappeared when the samples were treated with proteases before electrophoresis (Fig. 4*C*, lanes 3 and 4). This indicated that the observed band shift resulted from an interaction of VirD2 with the single-stranded DNA substrate. When a double-stranded T-DNA border fragment (Fig. 4*B*, lanes 1–5) or its 5′-end-labeled bottom strand (Fig. 4*B*, lanes 6–10) was used as probe, no retarded complex was detected. Incubation of the latter probe with VirD2 yielded a fast-migrating band on SDS/PAGE, the product of the VirD2-mediated nicking reaction. These results indicate that the band shift detected in SDS/polyacrylamide gels with the bottom-strand substrate resulted from the formation of a protein–DNA complex that consisted of VirD2 linked to the 5′ end of nicked single-stranded substrate DNA.

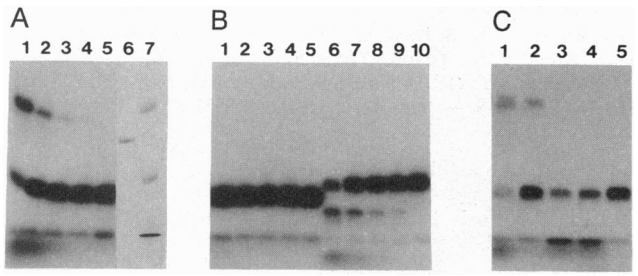

FIG. 4. After *in vitro* nicking VirD2 remains bound to the 5′ end of artificial T-strand substrates. (*A*) Substrate, 1.2 ng (14 fmol) of 3′-end-labeled bottom strand of T-DNA border fragment (Fig. 2, a template of 138 bases), was incubated with 2.0, 1.0, 0.5, or 0.25 μg of VirD2 protein (lanes 1–4) respectively, or without VirD2 (lane 5). After the nicking reaction the samples were denatured (95°C, 3 min) in the presence of 2% SDS and loaded on an SDS/10% polyacrylamide gel. Part of the gel stained by Coomassie blue shows 2 μg of VirD2 (lane 6), and molecular mass standards (lane 7; upper band is bovine serum albumin, 66.2 kDa; lower band is ovalbumin, 45 kDa). (*B*) VirD2 nicking assays were performed with 3′-labeled double-stranded T-DNA border fragment (lanes 1–5) and 5′-end-labeled bottom strand of the T-DNA border fragment (lanes 6–10). Samples 1–4 and 6–9 contained, respectively, 2.0, 1.0, 0.5, and 0.25 μg of VirD2, whereas VirD2 was omitted from samples 5 and 10. (*C*) The 3′-labeled bottom strand of the T-DNA border fragment was incubated with 2.0 μg (lanes 1, 3, and 4) or 0.5 μg (lane 2) of VirD2. Samples 3 and 4 were treated with 2 μg of proteinase K and 20 μg of Pronase, respectively, before denaturation and SDS/10% PAGE. Lane 5, sample without protein.

Similar VirD2 nicking assays were performed with a non-labeled T-strand template. After nicking, the samples either were treated with proteases or without protease treatment were subjected to 5′-end labeling by T7 polynucleotide kinase. Whereas both the intact substrate and the nicking product carrying the 3′ end of the VirD2 cleavage site were readily labeled at the 5′ end, no labeling of the nicking product carrying the 5′ end of the VirD2 cleavage site was observed (data not shown). This indicates that after nicking *in vitro*, the VirD2 protein blocks the 5′ end of artificial T-strands, probably by covalent linkage.

DISCUSSION

Some of the data described above are unexpected because they are different from the results obtained by *in vivo* studies of T-DNA border processing. With *Agrobacterium vir* gene mutants (8, 9) and *E. coli* strains expressing both VirD1 and VirD2 proteins (12, 33), it was demonstrated that VirD1 and VirD2 functions were both necessary and sufficient for cleavage of the T-DNA borders *in vivo*. It is important to note that all earlier studies of T-DNA border processing used natural plasmid templates that occur in supercoiled form *in vivo*.

Studies of plasmid relaxosomal complexes reconstituted *in vitro* showed that sequence-specific and DNA-strand-specific nicking of *oriT* sequences in double-stranded templates occurred efficiently only when the DNA templates were provided in a supercoiled form (4, 13–15). The analogy between the mechanism of T-DNA border processing and the *oriT* cleavage of RK2 or RSF1010 (5–20) and the fact that *in vitro* VirD1 and VirD2 are not sufficient for nicking of linear or supercoiled double-stranded DNA substrates suggest that *in vivo* VirD1 and VirD2 may act in concert with other proteins. In analogy to interactions among MobA, -B, and -C proteins of RSF1010 and among TraI, -J, and -H proteins of RK2, it is probable that the hypothetical T-DNA border processing complex ("T-DNA relaxosome") contains additional subunits, such as VirC1, VirD3, and/or VirD4, that facilitate the action of VirD1 and VirD2 on supercoiled substrates (4, 12, 34). By interacting with these proteins, VirD1 may induce local destabilization of the double helix by using the energy of the supercoil; this destabilization would lead to partial denaturation of the T-DNA border sequence. Such a single-stranded loop could then provide a substrate for VirD2. In support of this hypothesis, *in vivo* association of VirD2 with S1 nuclease-sensitive regions of a target plasmid DNA was demonstrated (35). Our results obtained *in vitro* with a purified VirD2 protein thus appear to specify the requirements for the strand-specific nicking reaction, which is only one step in the complex events leading to T-DNA border processing.

The data described here show that VirD2 catalyzes sequence- and strand-specific cleavage of single-stranded DNA templates at the 25-bp border sequences of the T-DNA. In contrast to TraI and MobA relaxosomal proteins, VirD2 has only a low ligase activity. This single-stranded-DNA ligase activity could not be enhanced by denaturation using high salt or temperature (data not shown). As is the case for the Mob protein of plasmid R1162 (16), the VirD2-mediated nicking reaction reaches a saturable equilibrium yielding on average 50% cleavage with artificial T-strand DNAs. VirD1 does not activate or reverse the VirD2-mediated nicking reaction on single-stranded or double-stranded templates. After nicking,

the VirD2 protein remains covalently, or at least very tightly, bound to the 5′ end of artificial T-strand DNAs at the third base of the T-DNA border sequence. Recent analysis of *virD2* point mutations affecting the nicking reaction in *E. coli* indicated that the Tyr^{29} is required for linkage of VirD2 to the T-strand (33). With the purified VirD2 protein, it will be possible to gain further insight into early steps of T-strand formation in *Agrobacterium*, as well as into the as yet obscure process of T-DNA integration in plants. Further, the *in vitro* produced T-strand provides a useful tool for the development of possibly efficient methods for artificial T-strand transformation of monocotyledonous and other crops that are recalcitrant to transformation by vectors harbored by *Agrobacterium* strains. These studies should also answer the question whether the difficulties in transformation of these recalcitrant plants are due to poor delivery of T-DNA to these plants or to the lack of molecular interactions allowing integration of T-DNA into their nuclear genome.

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