Role of the *Agrobacterium tumefaciens* VirD2 Protein in T-DNA Transfer and Integration

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VirD2 is one of the key *Agrobacterium tumefaciens* proteins involved in T-DNA processing and transfer. In addition to its endonuclease domain, VirD2 contains a bipartite C-terminal nuclear localization sequence (NLS) and a conserved region called ω that is important for virulence. Previous results from our laboratory indicated that the C-terminal, bipartite NLS and the ω region are not essential for nuclear uptake of T-DNA, and further suggested that the ω domain may be required for efficient integration of T-DNA into the plant genome. In this study, we took two approaches to investigate the importance of the ω domain in T-DNA integration. Using the first approach, we constructed a T-DNA binary vector containing a promoterless *gusA*-intron gene just inside the right T-DNA border. The expression of β -glucuronidase (GUS) activity in plant cells transformed by this T-DNA would indicate that the T-DNA integrated downstream of a plant promoter. Approximately 0.4% of the tobacco cell clusters infected by a wild-type *A. tumefaciens* strain harboring this vector stained blue with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-gluc). However, using an ω -mutant *A. tumefaciens* strain harboring the same binary vector, we did not detect any blue staining. Using the second approach, we directly demonstrated that more T-DNA is integrated into high-molecular-weight plant DNA after infection of *Arabidopsis thaliana* cells with a wild-type *A. tumefaciens* strain than with a strain containing a VirD2 ω deletion/substitution. Taken together, these data indicate that the VirD2 ω domain is important for efficient T-DNA integration. To determine whether the use of the T-DNA right border is altered in those few tumors generated by *A. tumefaciens* strains harboring the ω mutation, we analyzed DNA extracted from these tumors. Our data indicate that the right border was used to integrate the T-DNA in a similar manner regardless of whether the VirD2 protein encoded by the inciting *A. tumefaciens* was wild-type or contained an ω mutation. In addition, a mutant VirD2 protein lacking the ω domain was as least as active in cleaving a T-DNA border in vitro as was the wild-type protein. Finally, we investigated the role of various amino ac-

ids of the ω and bipartite NLS domains in the targeting of a GUS-VirD2 fusion protein to the nucleus of electroporated tobacco protoplasts. Deletion of the ω domain, or mutation of the 10-amino-acid region between the two components of the bipartite NLS, had little effect upon the nuclear targeting of the GUS-VirD2 fusion protein. Mutation of both components of the NLS reduced, but did not eliminate, targeting of the fusion protein to the nucleus.

Agrobacterium tumefaciens is a soilborne phytopathogen that causes crown gall tumors in plants (for recent reviews, see Gelvin 1992; Hooykaas and Beijersbergen 1994; Zupan and Zambryski 1995, 1997; Sheng and Citovsky 1996). The process of tumor formation involves the transfer from the bacterium and integration into the plant genome of a specific segment of the Ti (tumor inducing) plasmid, the T-(transferred) DNA (Chilton et al. 1977). T-DNA is flanked by 25-bp, imperfect, direct repeats called border sequences (Yadav et al. 1982; Slightom et al. 1985). Only the right border is required in *cis* for efficient T-DNA transfer (Wang et al. 1984; Peralta and Ream 1985; Jen and Chilton 1986). T-DNA encodes enzymes responsible for the biosynthesis of low-molecular-weight compounds termed opines (Montoya et al. 1977) as well as for enzymes involved in the synthesis of auxins and cytokinins (Akiyoshi et al. 1983; Schroder et al. 1983). Expression of these latter genes in transformed cells causes tumorous proliferation of the plant tissue. Virulence (*vir*) genes necessary for T-DNA processing and transfer lie elsewhere on the Ti plasmid. *vir* gene expression is induced by phenolic compounds produced by wounded plants (Stachel et al. 1985). The *vir* region of octopine-type Ti-plasmids is composed of at least eight complementation groups that include *virA*, *virB*, *virC*, *virD*, *virE*, *virF*, *virG*, and *virH* (Sheng and Citovsky 1996).

T-DNA processing involves the nicking of the T-DNA borders between nucleotides 3 and 4 (Albright et al. 1987; Wang et al. 1987) by the VirD1/VirD2 endonuclease (Yanofsky et al. 1986; Jayaswal et al. 1987; Stachel et al. 1987). This process results in the generation of single-stranded T-DNA molecules (T-strands) tightly associated with the VirD2 protein at the 5' end (Herrera-Estrella et al. 1988; Ward and Barnes 1988; Young et al. 1988; Howard et al.

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1989). These single-stranded DNA molecules are transferred to the plant cytoplasm (Tinland et al. 1994; Yusibov et al. 1994). It has been proposed that the T-strand is transferred as a protein-nucleic acid complex (the T-complex; Howard and Citovsky 1990). In addition to T-DNA and VirD2, this T-complex would contain the single-stranded DNA binding protein VirE2 (Gietl et al. 1987; Christie et al. 1988; Citovsky et al. 1988; Das 1988) that is believed to protect the T-DNA from nucleolytic activity (Christie et al. 1989). Recent evidence, however, suggests that VirE2 protein and the VirD2/T-strand complex may exit the bacteria separately (Binns et al. 1995; Sundberg et al. 1996).

The T-DNA border-specific endonuclease activity lies entirely within the N-terminal 228 amino acids of VirD2. This region and two very short regions near the C terminus are the only highly conserved domains of this protein (Steck et al. 1990). However, a region important for virulence resides in the C-terminal half of the protein. Deletion and insertion mutations in this region do not affect T-DNA border cleavage (Yanofsky 1986; Jayaswal et al. 1987; Steck et al. 1990). Subsequent experiments indicated that VirD2 and VirE2 proteins contain nuclear localization sequences (NLS) that presumably serve to direct the complexed T-strand to the plant nucleus (Herrera-Estrella et al. 1990; Citovsky et al. 1992; Howard et al. 1992; Shurvinton et al. 1992; Tinland et al. 1992; Rossi et al. 1993; Citovsky et al. 1994). VirD2 protein contains two putative NLS regions: an N-terminal, monopartite NLS (Herrera-Estrella et al. 1990; Tinland et al. 1992) and a C-terminal, bipartite NLS, rich in basic amino acids, resembling that found in *Xenopus laevis* nucleoplasmin (Robbins et al. 1991; Tinland et al. 1992). Although the N-terminal NLS can target a reporter protein to the nucleus (Herrera-Estrella et al. 1990; Tinland et al. 1992), mutagenesis experiments suggested that only the C-terminal NLS contributes to the targeting of T-strands to the nucleus (Shurvinton et al. 1992; Koukolikova-Nicola et al. 1993; Rossi et al. 1993). However, precise deletion of this C-terminal NLS only slightly (by 40%) reduced the virulence of *A. tumefaciens* (Shurvinton et al. 1992). The VirE2 single-stranded DNA binding protein, which contains two NLS (Citovsky et al. 1992), may therefore target T-DNA to host nuclei in the absence of the VirD2 NLS. Shurvinton et al. (1992) identified another region of VirD2, called ω , that was very important for virulence. The ω region consists of the five highly conserved penultimate amino acids DGRGG. These amino acids are not part of a known NLS motif, but their deletion resulted in a decrease in virulence by almost two orders of magnitude (Shurvinton et al. 1992).

We recently obtained evidence that tobacco cells infected by *A. tumefaciens* strains lacking the C-terminal NLS and the ω sequences can accumulate relatively high levels of T-DNA-encoded transcripts within 24 h after infection (Narasimhulu et al. 1996), despite the fact that a VirD2 ω deletion/substitution mutant strain is almost avirulent (Shurvinton et al. 1992). We therefore suggested that the ω region of VirD2 may be involved in T-DNA integration. We report here additional experiments that indicate that the ω region of VirD2 is involved in the efficiency, but not the pattern, of T-DNA integration. We additionally report the effect of various VirD2 NLS and ω mutations on T-DNA transfer and nuclear targeting.

RESULTS

A T-DNA-encoded *gusA* gene is transcribed early after infection of tobacco cells with *A. tumefaciens* VirD2 NLS and ω mutants.

To investigate the importance of various domains of the *A. tumefaciens* VirD2 protein in T-DNA transfer to the plant cell and T-DNA nuclear targeting, we used reverse transcription-polymerase chain reaction (RT-PCR) to detect early transcription of the T-DNA (Narasimhulu et al. 1996). We infected tobacco BY-2 suspension culture cells with various *A. tumefaciens* VirD2 mutant strains harboring the binary T-DNA vector pBISN2 (Narasimhulu et al. 1996). This binary vector contains a *gusA*-intron gene under the transcriptional control of a very strong synthetic promoter (composed of a trimer of the octopine synthase activating element affixed to the mannopine synthase 2' activating element plus minimal promoter; Ni et al. 1995). At various times after infection, we isolated total plant RNA and assayed for the expression of the processed *gusA* mRNA.

Figure 1A shows that tobacco cells infected by wild-type *A. tumefaciens* At822 first produced detectable *gusA* mRNA 18 h after infection. The amount of *gusA* mRNA (detectable as a 543-bp PCR product) gradually increased, peaked at 36 h, and subsequently declined. Figure 1C indicates that tobacco cells infected by *A. tumefaciens* At829 showed a similar pattern of *gusA* mRNA accumulation. This strain encodes a mutant VirD2 protein in which four of the five ω domain amino acids are replaced by two serines (Shurvinton et al. 1992). The results of quantitative PCR analysis (Narasimhulu et al. 1996) reported in Table 1 show that, at 36 h after infection, tobacco cells infected with the *virD2* ω deletion/substitution mutant accumulated 20% of the level of *gusA* mRNA of cells infected with a strain containing a wild-type *virD2* gene. These results were reproducible for sets of RNAs extracted from cells of two separate infections. These patterns and relative amounts of *gusA* mRNA accumulation closely resemble the values previously reported (Narasimhulu et al. 1996).

Figure 1B and Table 1 show that tobacco cells infected by *A. tumefaciens* At827, which contains a mutant *virD2* gene lacking both parts of the bipartite NLS, accumulate *gusA* mRNA to the same level as cells infected with the wild-type *A. tumefaciens* strain. This result indicates that the VirD2 C-terminal bipartite NLS is not essential for T-DNA nuclear transport. We suggest that NLS sequences in VirE2 can target T-strands to the nucleus efficiently. The relatively high level of *gusA* mRNA accumulation in tobacco cells infected with *A. tumefaciens* At827 correlates well with the relatively high level of tumorigenesis (60% of the wild-type value) on potato disks inoculated with an *A. tumefaciens* strain harboring the same NLS mutation (Shurvinton et al. 1992).

Finally, we infected tobacco cells with *A. tumefaciens* At823. This strain contains a large deletion in *virD2* (Δ NruI) that eliminates the C-terminal NLS and an additional 23 amino acids upstream of the NLS sequence. Shurvinton et al. (1992) showed that the virulence of this strain is extremely attenuated (1 to 4% the level of wild type). In accord with their result, we were unable to detect *gusA* mRNA in tobacco cells infected with this strain (Fig. 1D and Table 1). T-strands accumulate within the bacteria to approximately wild-type levels, however, in *A. tumefaciens* strains harboring this *virD2*

Δ NruI deletion (Shurvinton et al. 1992). Taken together, the data of Shurvinton et al. and the data presented here suggest that tumorigenesis by this mutant strain is blocked at an early step in the transformation process, most likely either at T-DNA transfer to the plant or T-DNA nuclear transport.

We sampled tobacco cells infected with the different *virD2* mutant strains at various times after infection and stained them with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-gluc) to determine the percentage of cell clusters that expressed β -glucuronidase (GUS) activity. Table 2 shows that when tobacco cell clusters were infected with strain At827 harboring the Δ NLS *virD2* mutation, the percentage of cell clusters showing detectable GUS staining eventually reached 0.12%, which is half the number of

GUS-positive cell clusters produced by infection with a wild-type strain. This level of GUS expression correlates well with the 60% level of virulence displayed by a similar *virD2* Δ NLS mutant strain (Shurvinton et al. 1992). However, tobacco cells infected with strain At829, harboring the ω deletion/substitution *virD2* allele, only occasionally (<0.01% of the cell clusters examined) expressed detectable GUS staining. These results agree with our previous observations (Narasimhulu et al. 1996) and indicate that, although early expression of the *gusA* gene in these cells is relatively efficient (20% that of the wild-type *virD2* gene), stable expression of GUS activity is rare. Our observations further suggest that infection of plant cells with strains harboring the ω deletion/substitution *virD2* allele does not result in the efficient, stable expression of T-DNA-encoded traits, perhaps because T-DNA transferred from these mutant strains cannot efficiently integrate into the plant genome. Finally, Table 2 shows that tobacco cells infected with strain At823 (Δ NruI *virD2* deletion) did not stain with X-gluc.

Long-term kinetics of GUS expression in tobacco cells infected with *A. tumefaciens* containing wild-type and ω -mutant *virD2* genes.

To investigate the importance of the VirD2 ω domain on the long-term stability of GUS expression in transformed tobacco cells, we infected BY-2 suspension cultures separately with *A. tumefaciens* At822 (wild-type *virD2*), At829 (ω deletion/substitution *virD2*), or At894 (lacking a Ti plasmid). Cocultivation for 48 h before adding antibiotics greatly increased the efficiency of infection, compared with 12-h cocultivations used in the experiments described above. Figure 2 shows the kinetics of GUS expression as determined by X-gluc staining. When tobacco cells were incubated with strain At822 containing the wild-type *virD2* gene, we detected blue staining of 2% of the cell clusters 1.5 days after the initiation of infection. The percentage of cell clusters showing detectable GUS staining increased rapidly and peaked at 4 to 5 days after the start of cocultivation. At this time, 20% of the cell clusters stained blue. After day 5, the percentage of blue-stained cell clusters declined rapidly, and at day 14 only 0.6%

Table 1. Quantitation of *gusA* mRNA by reverse transcription–polymerase chain reaction 36 h after infection

<i>Agrobacterium tumefaciens</i> strain	<i>virD2</i> allele	<i>gusA</i> mRNA (pg)/ total cell RNA (μ g)
At822	Wild type	0.25
At827	Δ NLS	0.25
At829	$\Delta\omega$	0.05
At823	Δ NruI	Not detectable

Table 2. Percentage of tobacco BY-2 cell clusters exhibiting β -glucuronidase (GUS) activity

<i>Agrobacterium tumefaciens</i> strain containing pBISN2	<i>virD2</i> allele	Days after infection				
		2	2.5	3	4	5
At822	Wild-type	0.04	0.12	0.13	0.23	0.23
At827	Δ NLS	0.00	0.05	0.03	0.13	0.12
At829	$\Delta\omega$	0.00	0.00	0.00	<0.01	>0.00 ^a
At823	Δ NruI	0.00	0.00	0.00	0.00	0.00

^a Blue-stained cells were occasionally observed.

Fig. 1. Early expression of a *gusA* gene in tobacco BY-2 cells after infection by *Agrobacterium tumefaciens* *virD2* wild-type and mutant strains. Tobacco suspension cell cultures were infected with different *A. tumefaciens* strains. After 0, 18, 24, 36, or 48 h, RNA was isolated and analyzed for the presence of processed *gusA* transcripts by reverse transcription–polymerase chain reaction (RT-PCR) as described in Materials and Methods. **A.** *tumefaciens* (A) At822, (B) At827, (C) At829, (D) At823. λ HindIII, length standards of bacteriophage λ DNA digested with HindIII; β -glucuronidase (GUS)-intron, PCR amplification of a 732-bp *gusA*-intron gene.

of the cell clusters stained blue. After 14 days and until 42 days (the last time point investigated), the percentage of blue-stained cell clusters stabilized at 0.4 to 0.6%.

Figure 2 also shows that when we infected tobacco cells with the *virD2* ω mutant strain At829, the percentage of GUS-stained cell clusters also peaked at 4 days, when 4% of the cell clusters were stained blue. The percentage of cell clusters stained blue then decreased until, by 12 days after infection, GUS staining was almost undetectable. We obtained similar results with *A. thaliana* cell cultures (data not shown). Both tobacco and *A. thaliana* cell cultures infected with *A. tumefaciens* At894 (lacking a Ti plasmid) did not show any blue staining (data not shown). For each of the samples taken for the experiment described above, we observed at least 10,000 cell clusters.

Our results indicate that after infection of tobacco cells with the wild-type strain At822, 2 to 3% of the cell clusters that initially (4 day time point) showed transient GUS staining ultimately showed stable GUS expression. Tobacco cells in-

Fig. 2. Kinetics of β -glucuronidase (GUS) expression in tobacco BY-2 suspension cells. Tobacco suspension cell cultures were infected with *Agrobacterium tumefaciens* strains containing either the wild-type *virD2* (At822) or the ω mutant *virD2* (At829) gene. Cells were sampled at various times after infection, stained with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-gluc), and the percentage of cell clusters staining blue was determined. Open box, *A. tumefaciens* At822. Open diamond, *A. tumefaciens* At829.

fectured with the *virD2* ω mutant initially showed only 20% of the number of blue-stained cell clusters showed by BY-2 cells infected with the wild-type strain (a number consistent with the RT-PCR and X-gluc staining data presented in Figure 1, Table 1, and Narasimhulu et al. 1996). However, we could not detect stable GUS staining in these cells. These results indicate that cells infected by this *virD2* ω mutant cannot stabilize GUS expression and further suggest that the ω region of VirD2 is important for T-DNA integration.

A wild-type, but not an ω mutant, *virD2* *A. tumefaciens* strain can induce GUS expression in tobacco cells transformed by a "promoter trap" T-DNA.

We constructed a T-DNA binary vector, pKM1, that could direct the expression of GUS activity in plants only after integration of the T-DNA downstream of a plant promoter. pKM1 contains a T-DNA right border sequence adjacent to the 5' end of a promoterless *gusA*-intron gene. This plasmid also contains a luciferase(*luc*)-intron gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Figure 3 presents a map of pKM1. We mobilized this plasmid into *A. tumefaciens* to generate strains At899 (containing a wild-type *virD2* gene) and At898 (containing a *virD2* ω deletion/substitution mutation), and used these strains to inoculate tobacco BY-2 cell cultures.

Table 3 shows that only tobacco cells transformed by *A. tumefaciens* At899 expressed GUS activity. Approximately 0.32 to 0.40% of these cell clusters stained blue with X-gluc. Cells infected with the *virD2* ω mutant strain At898 did not express GUS activity. For each of the samples taken for the experiment described above, we observed at least 5,000 cell clusters. To test whether strain At898 transferred T-DNA to tobacco nuclei, we assayed expression of the T-DNA-encoded *luc* gene by RT-PCR. Figure 4 shows that luciferase transcripts with the intron spliced out accumulated to a maximal extent 36 h after infection, after which their levels declined. The pattern of *luc* mRNA accumulation closely resembled that of *gusA* mRNA accumulation shown in Figure 1C.

Our results indicate that although an *A. tumefaciens* strain that contains this *virD2* ω mutation could transfer T-DNA to tobacco nuclei and transiently express a *luc* gene that contains a promoter, it could not do so with a *gusA* gene that required integration into the plant genome for expression. These data further suggest that the ω domain of VirD2 is required for efficient T-DNA integration into plant DNA.

Fig. 3. Map of the promoter trap plasmid pKM1. RB, T-DNA right border; LB, T-DNA left border; intron, potato ST-LS1 gene intron (Vancanneyt et al. 1990); GUS (β -glucuronidase), *gusA* coding region; OCS 3', octopine synthase polyadenylation signal sequence; P_{35S}, cauliflower mosaic virus (CaMV) 35S promoter; LUCIFERASE, *luc* coding region; NOS 3', nopaline synthase polyadenylation signal sequence.

An *A. tumefaciens* *virD2* ω mutant strain is deficient in T-DNA integration.

We infected *A. thaliana* suspension cell cultures separately with *A. tumefaciens* At822 (wild-type *virD2*), At829 (ω deletion/substitution *virD2* mutant), or At894 (no Ti plasmid). After 2 days of cocultivation, we added antibiotics to the medium to kill the bacteria and continued to grow the plant cells in the presence of phytohormones and antibiotics. Because of the presence of phytohormones in the medium, tumor cells would not outgrow the uninfected cells. We transferred these cells several times for a period of greater than 1 month to assure that all *A. tumefaciens* cells were killed, and that DNA from any remaining dead cells would be diluted from the culture. After 40 days of growth, we isolated high-molecular-weight DNA from these cultures and assayed for the integration of the T-DNA-encoded *gusA* gene by DNA gel blot analysis. Figure 5A shows that T-DNA integrated into high-molecular-weight plant DNA of *A. thaliana* cells infected by strain At822 containing a wild-type *virD2* gene. However, we could not detect T-DNA integrated into the genome of *A. thaliana* cells infected with the *virD2* ω mutant strain At829. As expected, we could not detect T-DNA integrated into the genome of *A. thaliana* cells infected with strain At894 lacking a Ti plasmid, or in uninfected cells. In order to assure that equal amounts of DNA were loaded onto each gel lane, we washed the *gusA* probe from the membrane and rehybridized the blot with an *A. thaliana* phenylalanine ammonia-lyase (PAL) gene. Figure 5B shows that equal amounts of DNA were present in each gel lane. These data show that plant cells infected with an *A. tumefaciens* strain lacking the ω domain of VirD2 do not efficiently integrate T-DNA into their genomes.

A VirD2 ω mutation does not affect the pattern of T-DNA integration.

Although *A. tumefaciens* strains harboring the *virD2* ω deletion/substitution mutation are severely attenuated in virulence, they are not avirulent. Shurvinton et al. (1992) showed that such a strain was able to induce crown gall tumors on potato disks, but with only 3.3% the efficiency of a wild-type strain. To investigate whether this ω mutation altered the pattern of T-DNA integration, we incited tumors on tobacco leaf disks with strains At747 (containing a wild-type *virD2* gene) and At746 (containing the ω deletion/substitution *virD2* gene). Infection of tobacco leaf disks with strain At746 resulted in 0.06 tumors per disk, compared with 2.3 tumors per disk for strain At747.

When T-DNA integrates into plant DNA, the right junction between bacterial DNA and plant DNA almost always occurs

precisely at the base where VirD2 nicks the right border; in contrast, the left junction between bacterial and plant DNA frequently does not occur within the left border sequence (Tinland and Hohn 1993; Tinland et al. 1995; Rossi et al. 1996). We therefore used the PCR to analyze T-DNA sequences near the right border. First, however, we used the PCR and primers specific for the *tms* oncogenes (Yusibov et al. 1994) to assure that all calli contained T-DNA. Figure 6C shows that all tumorous calli contained this oncogene. We then analyzed the tumors for the presence of the *picA* gene. *picA* is contained on the *A. tumefaciens* chromosome (Rong et al. 1990). The presence of *picA* sequences in the tumor DNA would indicate that this plant DNA contained DNA from contaminating *A. tumefaciens* cells. We previously showed that the sensitivity of detection of *picA* sequences was at least as great as our ability to detect *tms* sequences with this technique (Yusibov et al. 1994). Figure 6D shows that we could not detect *picA* sequences among the tumors investigated. Therefore, T-DNA border sequences detected in tumor DNA resulted from integrated T-DNA and not from contaminating bacterial DNA.

To determine whether the right T-DNA/plant DNA junction extended to the VirD2 nick site in the right border or whether T-DNA sequences at the right border junction had been truncated as a result of the VirD2 ω mutation, we synthesized primers homologous to the T-DNA at various distances from this border, as shown in Figure 6A. Primer rb1 extends leftward from the nucleotide immediately to the left of the VirD2 nick site, rb2 contains sequences within the T-DNA 35 bp from the RB, rb3 has sequences within the T-DNA still further from the RB, etc. Primer rb6 is homologous to T-DNA sequences beginning 869 bp to the left of the right border and extends in the opposite orientation from primers rb1 to rb5 (Fig. 6A).

Fig. 4. Early expression of the luciferase (*luc*) gene encoded by the promoter trap vector pKM1 in tobacco BY-2 cells infected by *Agrobacterium* strain At898. Tobacco suspension cell cultures were infected with *Agrobacterium* strain At898 (ω mutant *virD2*) harboring the promoter-trap binary vector pKM1. RNA was isolated from samples taken at various times after infection and subjected to reverse transcription-polymerase chain reaction (RT-PCR) with primers that flank the *luc*-intron region. Amplification products (623 bp for the processed mRNA) were subjected to electrophoresis through a 1.2% agarose gel, blotted onto a nylon membrane, and hybridized with a *luc* gene probe. Luc intron, amplification of the *luc*-intron gene (812 bp amplification product) from pKM1. Water, no RNA added to the amplification mix.

Table 3. Percentage of tobacco BY-2 cell clusters exhibiting β -glucuronidase (GUS) activity

Days after infection	<i>Agrobacterium tumefaciens</i> strain containing pKM1	
	At899 (wild-type <i>virD2</i>)	At898 (ω mutant <i>virD2</i>)
2	0.04 (2) ^a	0.00 (0)
3	0.32 (16)	0.00 (0)
4	0.36 (18)	0.00 (0)
5	0.40 (20)	0.00 (0)
6	0.36 (18)	0.00 (0)
8	0.36 (18)	0.00 (0)

^a Numbers in parenthesis indicate number of blue-stained cell clusters from a total of 5,000 cell clusters observed.

PCR analysis of 27 tumors that were produced by *A. tumefaciens* At747 with wild-type *virD2* resulted in the amplification of the expected 869-bp fragment with primers rb1 and rb6. Of the 43 tumors produced by the *virD2* ω mutant strain At746, all also showed this amplification product (Fig. 6B). These results indicate that, within the limitations of this PCR technique, the *virD2* ω mutant preserved the right T-DNA end upon integration as well as the wild-type strain did. Thus, the *virD2* ω deletion/substitution mutation affects the efficiency but not the precision of T-DNA integration.

A *VirD2* ω deletion mutant protein cleaves the right border efficiently and correctly in vitro.

We purified wild-type *VirD2* protein and a mutant protein, $\Delta\omega$ *VirD2* (which lacks the five ω amino acids plus the terminal threonine residue), from *Escherichia coli* and tested these proteins for their ability to cleave a DNA fragment containing a T-DNA right border sequence in vitro. Figure 7 shows that each protein cleaved a 530 nucleotide, single-stranded DNA segment (uniformly labeled with ^{32}P) between nucleotides 3 and 4 within the right T-DNA border, resulting in the production of a 460 nucleotide, labeled fragment. The $\Delta\omega$ *VirD2* protein was at least as active in this cleavage as the wild-type *VirD2* protein was. We con-

clude that the ω domain of *VirD2* is not required for in vitro cleavage of a T-DNA border sequence.

Mutation analysis of the *VirD2* bipartite NLS and the ω region in nuclear transport.

The C-terminal region of *VirD2* contains a bipartite NLS upstream of the ω domain that, when fused to the C terminus of GUS, can target the fusion protein to the nucleus of plant and yeast cells (Howard et al. 1992; Tinland et al. 1992; Rossi et al. 1993). Previous analyses of this NLS involved the generation of mutations that altered the charge of the protein. In addition, the role of the ω domain in nuclear targeting was unknown because all GUS-*VirD2* NLS fusions previously tested contained ω . We therefore investigated further the importance of amino acids in this region of *VirD2* in targeting a GUS fusion protein to tobacco nuclei. We generated several mutations affecting the C-terminal region of *VirD2*. Figure 8 shows the amino acid sequence of this region of *VirD2*, and the sequences of the various mutant proteins. In all polypeptides with mutant NLS domains, amino acid substitutions did not change the number or polarity of charged residues within the NLS domain. We created substitution mutations in each half of the bipartite NLS, in both halves of the NLS, in the spacer region between the two NLS components, and in the

Fig. 5. Effect of the *virD2* ω region on T-DNA integration in *Arabidopsis thaliana* suspension cells. *A. thaliana* suspension cells were cocultivated with *Agrobacterium tumefaciens* strains containing the wild-type *virD2* gene (At822), an ω mutant *virD2* gene (At829), or lacking a tumor-inducing (Ti) plasmid (no *vir* genes, At894). After 2 days, the cells were washed and resuspended in medium containing antibiotics to kill the bacteria. High-molecular-weight plant DNA was extracted from cells 42 days after infection, or from uninfected cells. Undigested DNA (5 μg) was blotted onto a nylon membrane and hybridized with (A) the *gusA* gene probe, (B) an *A. thaliana* phenylalanine ammonia-lyase (PAL) gene probe. kb, DNA size markers of bacteriophage λ DNA digested with *Hind*III.

entire region containing both bipartite NLS components. We also tested a deletion mutation precisely eliminating the five ω amino acids but maintaining the wild-type NLS intact. We placed the *gusA-virD2* fusion genes under the control of the CaMV 35S promoter and electroporated the constructions into tobacco protoplasts to study the role of each domain in the nuclear transport of GUS-NLS fusion proteins. We quantitated nuclear targeting by calculating the percentage of tobacco cells that, when stained with X-gluc, showed GUS activity exclusively in the nucleus.

Figures 9 and 10 indicate that when the VirD2 NLS was not fused to the GUS protein, only 8% of the cells showed GUS activity in the nucleus; 92% of the cells showed GUS activity exclusively in the cytoplasm. When the wild-type VirD2 NLS was fused to the GUS protein, 87% of the cells localized GUS activity exclusively in the nucleus. When either of the NLS domains was individually mutated, the percentage of cells showing exclusive nuclear localization of GUS activity was reduced to approximately 70%. Mutation of both components of the bipartite NLS further reduced exclusive nuclear localization of GUS activity to 27% of the cells. Mutation of the entire region containing both components of the C-terminal bipartite NLS reduced nuclear localization of GUS activity to 10% of the cells, a number equivalent to that when no NLS was affixed to the GUS protein. Mutation of three of the 10

amino acids in the spacer region between the two components of the bipartite NLS had virtually no effect upon nuclear targeting. Similarly, complete deletion of the ω region did not affect the nuclear localization of GUS activity. We conducted electroporation experiments for each construction at least three times, and counted 300 to 600 blue-stained cells for each construction.

Our analysis indicates that the ω domain of VirD2 is not involved in nuclear targeting. As shown by others (Howard et al. 1992), each component of the bipartite NLS by itself has some nuclear targeting activity.

DISCUSSION

VirD2 contains a number of domains involved in T-DNA processing, nuclear targeting, and integration. The N-terminal half of the protein contains an endonuclease activity and, along with VirD1 protein, is sufficient to cleave T-DNA borders in both *E. coli* and *A. tumefaciens* (Yanofsky et al. 1986; Jayaswal et al. 1987; Stachel et al. 1987; Filichkin and Gelvin 1993). The 5' end of the resulting single-stranded DNA (the T-strand) becomes linked to VirD2 through a tyrosine residue near the amino terminus of the protein (Herrera-Estrella et al. 1988; Ward and Barnes 1988; Young and Nester 1988; Howard et al. 1989; Vogel and Das 1992). Although *A. tumefaciens* strains harboring a mutant VirD2 protein lacking the C-terminal half of the molecule accumulate T-strands upon induction with acetosyringone (Stachel et al. 1987), they are avirulent (Stachel and Nester 1986; Koukolikova-Nicola et al. 1993). This result indicates that the C-terminal portion of

Fig. 6. Polymerase chain reaction (PCR) amplification of DNA from tumors generated by infection of tobacco with wild-type and Δ *virD2* mutant *Agrobacterium tumefaciens* strains. DNA was isolated from tumors generated by infection of tobacco leaf disks with the ω mutant *virD2* *A. tumefaciens* At746. DNA was subjected to the PCR with various primer combinations. Amplified products were analyzed by agarose gel electrophoresis. Lanes 1 to 12, the amplified DNA from different tumors. wt, amplification product from nontransformed W38 tobacco DNA; -, amplification product from a sample lacking DNA; +, amplification product from total DNA of *A. tumefaciens* At747; λ , molecular size markers from bacteriophage λ digested with *Hind*III. **A**, Position and orientation of primers rb1 to rb6 in the T-DNA T_L right border region of pTiA6. 0, position of nick site in the border. Other numbers (-x), distance (in nucleotides) of 5' end of primers from right border nick site. **B**, Amplification products with rb1 and rb6 primers. **C**, Amplification products with *tms* primers. **D**, Amplification products with *picA* primers.

Fig. 7. In vitro cleavage of a T-DNA right border with wild-type VirD2 and $\Delta\omega$ VirD2 proteins. A uniformly labeled, 530-base, single-stranded DNA fragment containing the right T-DNA border sequence was incubated with various amounts of either wild-type VirD2 or the $\Delta\omega$ mutant VirD2 protein. After electrophoresis through a 4% polyacrylamide gel, the 460-base cleavage product was detected by autoradiography. **A**, Wild-type VirD2 protein. **B**, $\Delta\omega$ VirD2 protein.

VirD2 contains information essential for the later processes of *A. tumefaciens*-mediated transformation. These processes may include T-strand export from the bacterium to the plant cell, nuclear targeting of the T-DNA, and/or T-DNA integration into the plant genome.

VirD2 contains an N-terminal, monopartite NLS that, when linked to a reporter protein, can direct the fusion protein to the nucleus of plant and yeast cells (Herrera-Estrella et al. 1990; Tinland et al. 1992). However, this NLS does not contribute to targeting of the T-DNA to the nucleus (Shurvinton et al. 1992; Rossi et al. 1993), most likely because it is occluded by the T-strand that is attached to the tyrosine residue near this NLS. VirD2 also contains a C-terminal, bipartite NLS and a region called ω . The ω domain consists of five penultimate, highly conserved amino acids and is not part of a known NLS motif. *A. tumefaciens* strains harboring a ω deletion/substitution mutation or some C-terminal NLS mutations are either avirulent or severely attenuated in virulence (Stachel and Nester 1986; Shurvinton et al. 1992; Koukolikova-Nicola et al. 1993). We have investigated which step(s) in the transformation process are blocked by these mutations.

The C-terminal NLS and ω sequences of VirD2 are not essential for T-DNA transfer to the plant cell or nuclear targeting.

Shurvinton et al. (1992) showed that precise deletion of both components of the VirD2 C-terminal NLS reduced the virulence of *A. tumefaciens* on potato disks by approximately 40%. Using the identical NLS deletion in a strain harboring the *gusA*-intron plasmid pBISN2, we showed that, by 4 days after infection, the percentage of tobacco cell clusters stained blue with X-gluc was approximately 50% of that seen when a strain harboring a wild-type *virD2* gene was used (Table 2). Moreover, when we used a sensitive RT-PCR assay to monitor transcription of the *gusA*-intron gene in tobacco cells only 36 h after infection, we detected the same level of processed *gusA* mRNA with the Δ NLS mutant as we did with a wild-type strain (Fig. 1, Table 1). These results indicate that the T-DNA transfer and nuclear targeting efficiencies of this Δ NLS and the wild-type VirD2 proteins are (within a factor of 2) approximately the same. Although we do not know whether X-gluc staining of tobacco cells 4 to 5 days after infection represents expression of integrated or nonintegrated T-DNA molecules, our results indicate that the VirD2 C-terminal NLS is

not essential for either T-DNA transfer or nuclear targeting. We suggest that deletion of the C-terminal NLS of VirD2 may be compensated by the NLS domains within VirE2 protein molecules that presumably coat T-strands in the plant cell (Citovsky et al. 1992).

Because deletion of the NLS domains did not markedly affect T-DNA transfer or nuclear targeting, we investigated the effect of larger deletions of this region of VirD2. When we used an *A. tumefaciens* strain containing the Δ NruI mutation of *virD2*, in which the C-terminal NLS domain is deleted along with 23 amino acids upstream, we did not detect *gusA* transcripts or X-gluc staining of infected tobacco cells (Fig. 1, Tables 1 and 2). These results are consistent with the data of Shurvinton et al. (1992), who showed that an *A. tumefaciens* strain harboring this same Δ NruI *virD2* mutation showed extremely low virulence. T-DNA processing in this strain is normal (Shurvinton et al. 1992). These results indicate that

Fig. 9. Nuclear localization of β -glucuronidase (GUS) activity in tobacco protoplasts electroporated with wild-type and mutant *gusA-virD2* fusion genes. Tobacco protoplasts were electroporated with *gusA* plasmids either lacking the VirD2 bipartite nuclear localization sequence (NLS) or containing wild-type or mutant VirD2 NLS and ω regions. Cells were stained with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-gluc) 20 h after electroporation, and the percentage of cells showing GUS activity exclusively in the nucleus was determined.

	NLS I	NLS II	ω
	KRPR	RKRAR	DGRGG
W.T. NLS	...QPEASPKRPRDRHDGELGGRKRARGNRRDDGRGGT		
MUT.NLS I	...QPEAS <u>WKFKK</u> DRHDGELGGRKRARGNRRDDGRGGT		
MUT.NLS II	...QPEASPKRPRDRHDGELG <u>WKFKK</u> GNRRDDGRGGT		
MUT.NLS I+II	...QPEAS <u>WKFKK</u> DRHDGELG <u>WKFKK</u> GNRRDDGRGGT		
MUT.SPACER	...QPEASPKRPRDRH <u>NWGL</u> GGRKRARGNRRDDGRGGT		
MUT.TOTAL NLS	...QPEA <u>YAERLHVRLRFKERVRETR</u> GNRRDDGRGGT		
Δω	...QPEASPKRPRDRHDGELGGRKRARGNRRD T		

Fig. 8. Amino acid substitution mutations within C-terminal region of VirD2 protein. W.T. NLS, sequence of C-terminal region of wild-type VirD2 protein. Below this sequence are the sequences of the mutant VirD2 proteins. Amino acids altered from the wild-type sequence are underlined. In $\Delta\omega$, the five amino acids of the ω domain are deleted. Amino acids defining the NLS I, NLS II, and ω domains are noted above the wild-type sequence.

Fig. 10. Cytological localization of β -glucuronidase (GUS) activity in tobacco protoplasts electroporated with wild-type and mutant *gusA-virD2* fusion genes. Tobacco protoplasts were electroporated with *gusA* plasmids either lacking the VirD2 bipartite nuclear localization sequence (NLS), or containing wild-type or mutant VirD2 NLS and ω regions. Cells were stained with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-gluc) 20 h after electroporation and visualized with a phase contrast microscope at $\times 400$ magnification.

deletion of this region inhibits transformation at a very early step. This deletion may alter the structure of VirD2 protein such that it can still nick the T-DNA border but may fail to pass through the VirB channel or the nuclear pore.

An *A. tumefaciens* strain containing an ω deletion/substitution mutation exhibits very low virulence compared with that of a strain containing the wild-type *virD2* gene (Shurvinton et al. 1992). Our GUS staining data (Table 2) were consistent with these observations. X-gluc staining of tobacco cells infected with this *virD2* ω mutant resulted in only a few blue cell clusters. Although both the virulence and the ability to transform plant cells to express GUS activity stably were very low with this mutant strain, RT-PCR analysis showed relatively high (20% of wild type) transient levels of *gusA* mRNA (Fig. 1, Table 1, and Narasimhulu et al. 1996). However, the presence of *gusA* transcripts in infected tobacco cells was transient (Fig. 1 and Narasimhulu et al. 1996). Thus, the ω region is not essential for T-DNA transfer or nuclear targeting. Wild-type levels of targeting of a GUS-VirD2 $\Delta\omega$ fusion protein to tobacco nuclei (Fig. 9) corroborate this result. The substitution of two serine residues for four ω amino acids may alter the conformation of VirD2 protein. The fivefold reduction (Table 1) in the transient level of *gusA* transcripts in tobacco cells infected by a strain harboring this ω mutation, compared with the wild-type *virD2* gene, is consistent with our suggestion that the conformation of VirD2 may additionally be important for T-DNA transfer and/or nuclear targeting. However, the extremely low virulence of this particular *virD2* ω mutation may be explained by the probable involvement of the VirD2 ω domain in T-DNA integration. We took two approaches to test this hypothesis.

The ω region of VirD2 is involved in T-DNA integration.

When tobacco cells were infected with an *A. tumefaciens* strain that contained the *virD2* ω deletion/substitution mutation, *gusA* transcripts initially accumulated with almost the same kinetics as when the plant cells were infected with a wild-type strain harboring pBISN2 (Fig. 1 and Narasimhulu et al. 1996). However, the level of *gusA* mRNA detected 36 h after infection with the ω mutant strain was only 20% of that seen when the wild-type strain was used (Table 1 and Narasimhulu et al. 1996). Similarly, the number of tobacco cell clusters stained blue 5 days after infection with an ω mutant *virD2* strain was approximately 20% of that with the wild-type *virD2* strain (Fig. 2). The percentage of cell clusters stained blue with X-gluc is lower in Table 2 than in Figure 2 because the infection protocol differed between these two experiments. To obtain the data shown in Figure 2, we cocultivated the bacteria and tobacco cells 48 h before killing the bacteria with antibiotics, whereas we cocultivated the bacteria and plant cells for only 12 h to obtain the data for Table 2. Cocultivation for longer times increased the percentage of infected tobacco cells, allowing us to quantitate better the effect of the ω mutation on transformation. The transient expression of GUS activity and *gusA* mRNA with the ω mutant strain was relatively high compared with the virulence of this strain (Shurvinton et al. 1992). However, the ω *virD2* mutant strain was much less efficient than the wild-type strain in stabilizing GUS activity after 8 days of infection.

Our first approach to test the hypothesis that the ω region of VirD2 is involved in T-DNA integration used a “promoter

trap” vector in which we transformed a promoterless *gusA* gene (Fig. 3) into tobacco cells. The rationale for this experiment was that GUS expression would occur only after integration of the T-DNA downstream of a plant promoter. “Promoter trap” vectors have been used by a number of scientists to investigate both plant gene expression and the integration preferences of T-DNA (Koncz et al. 1989; Ott and Chua 1990; Goldsbrough and Bevan 1991; Fobert et al. 1994). Table 3 shows that 0.3 to 0.4% of the cocultivated tobacco cells stained blue with X-gluc when the T-DNA from the vector pKM1 was delivered from wild-type *A. tumefaciens* cells. However, we could not detect GUS activity when this T-DNA was transferred from a *virD2* ω mutant strain. To show that the “promoter trap” T-DNA could be transferred to tobacco nuclei, we determined that transcription of a *luc* gene under the control of a CaMV 35S promoter occurred when delivered from within the same T-DNA as the promoterless *gusA*-intron gene in the ω mutant strain (Fig. 4). We could not directly visualize the RT-PCR amplification product of the *luc* gene from the cells infected with the *A. tumefaciens* strain containing either wild-type or an ω mutant VirD2. It was therefore necessary for us to detect transcription of the *luc* gene by hybridization. The likely reason for this result was that the transcription of the *luc* gene was driven by a CaMV 35S promoter, while our previous RT-PCR experiments involved amplification of a *gusA*-intron gene driven by a “super-promoter” that is approximately 100 times stronger than the CaMV 35S promoter (Ni et al. 1995). These results further suggested that the ω region of VirD2 was primarily involved in T-DNA integration, but not greatly involved in T-DNA transfer and nuclear targeting. Interestingly, two other *virD2* mutant alleles resulted in a similarly weak decrease in T-DNA transfer efficiency, but had no influence on the efficiency or precision of T-DNA integration (Bravo-Angel et al. 1998).

We therefore directly tested the integration of T-DNA into high-molecular-weight plant DNA after infection of *A. thaliana* suspension cells with *A. tumefaciens* strains harboring wild-type or ω mutant *virD2* alleles. The genome size of *A. thaliana* is approximately 3% that of tobacco (Arumuganathan and Earle 1991). Thus, it was easier to detect the integration of T-DNA into the genome of *A. thaliana* than into that of tobacco. Figure 5 shows that T-DNA integrated into *A. thaliana* high-molecular weight DNA to a much greater extent when the plant cells were infected with an *A. tumefaciens* strain harboring a wild-type *virD2* gene than with a strain containing a ω mutant *virD2* gene. These data directly demonstrate that the ω domain of VirD2 protein is involved in T-DNA integration.

A *virD2* ω mutant cleaves and integrates T-DNA precisely.

Tinland et al. (1995) showed that changing arginine residue 129 of VirD2 to a glycine (R129G) resulted in the alteration of a number of properties of the protein. Although this mutant VirD2 could efficiently nick a T-DNA border and bind to the 5' end of the T-strand with the same precision as the wild-type protein in vitro, the mutant protein processed T-DNA inefficiently in *A. tumefaciens*. Hence, the T-DNA transfer efficiency to tobacco cells, as determined by GUS expression, was greatly reduced. By measuring the frequency of transformation of tobacco to kanamycin-resistance, Tinland et al. (1995) calculated that the T-DNA integration frequency (the

ratio of transformation efficiency to transfer efficiency) was not altered with the mutant *virD2* strain. However, use of the R129G *virD2* gene altered the precision with which T-DNA at the right border nick site was ligated to plant DNA. These authors therefore concluded that VirD2 played a role in T-DNA integration into the plant genome.

Because the VirD2 ω deletion/substitution mutation described above altered the efficiency of T-DNA integration, we investigated whether this mutant VirD2 protein cleaved a T-DNA border precisely in vitro, and whether the in vivo precision of use of the right T-DNA border to join T-DNA to tobacco DNA was normal. Our results (Fig. 7) showed that the ω mutant VirD2 cleaved a right border efficiently and correctly in vitro. To determine the pattern of T-DNA integration in those few tobacco tumors that we obtained with the *virD2* ω mutant, we amplified from tumor DNA the T-DNA right border region (Tinland and Hohn 1993; Koncz et al. 1994) with different primer combinations (Fig. 6A). Our results showed that in all tumors tested, whether they were incited by *A. tumefaciens* strains harboring a wild-type or ω mutant *virD2* gene, we could amplify a segment of T-DNA whose junction with plant DNA was located very close to the 5' VirD2 nick site. From these results we conclude that, although the VirD2 ω mutation affected the efficiency of T-DNA integration, it did not affect the precision of use of the right border during integration. Thus, the ω mutation that we investigated had the opposite effect of the R129G mutation: This mutation of ω resulted in a low frequency of T-DNA integration, but those few integration events joined T-DNA with plant DNA at or very near the right border nick site. However, the R129G mutation resulted in a relatively high frequency of imprecise T-DNA integration.

The ω region of *virD2* does not have nuclear targeting activity.

The C-terminal region of VirD2 contains an NLS that can target both T-DNA and fusion proteins to plant nuclei (Howard et al. 1992; Shurvinton et al. 1992; Tinland et al. 1992; Koukolikova-Nicola et al. 1993; Rossi et al. 1993). To determine the relative contribution to nuclear targeting of various peptide domains in the NLS and ω regions of VirD2, we constructed chimeric genes in which various mutant VirD2 peptides were fused to the C terminus of GUS protein (Fig. 8). We took care to preserve the basic charge of the mutagenized domains. Electroporation of these genes into tobacco protoplasts, followed by staining with X-gluc, revealed the targeting activity of each mutant protein (Figs. 9 and 10). Our results indicated that mutagenesis of either one of the two bipartite NLS motifs resulted in only a slight decrease in nuclear targeting activity. These results are similar to those obtained by Howard et al. (1992) and Shurvinton et al. (1992). Mutation of both bipartite NLS motifs resulted in a further reduction, but not elimination, of nuclear targeting activity. Shurvinton et al. (1992) showed that deletion of both NLS motifs severely attenuated virulence of the resulting *A. tumefaciens* strain when inoculated on tobacco. However, infection of potato disks with this VirD2 NLS mutant only reduced virulence by approximately 40%. Thus, the importance of the VirD2 NLS to virulence may depend upon the species of host plant. In order to eliminate nuclear targeting activity, we had to mutate a larger, 20 amino acid domain containing both NLS

motifs and the intervening "spacer" amino acids. Mutation of only the spacer region between the two components of the bipartite NLS did not affect nuclear targeting of the fusion protein.

The ω domain adjoins the C-terminal NLS of VirD2, and because of this close proximity to the NLS, all GUS-VirD2 NLS fusions tested to date have included the ω domain (Howard et al. 1992; Tinland et al. 1992; Rossi et al. 1993). Thus, the possible role of ω in nuclear targeting was not tested previously. It was especially important to test for such a role in light of our observation that elimination of the ω domain reduced transient expression of a *gusA* gene transferred to tobacco. Elimination of ω from the GUS-VirD2 fusion protein did not reduce nuclear localization of this protein. Thus, the ω domain of VirD2 is not essential for targeting the fusion protein to plant nuclei.

The results of this study indicate that the ω domain of VirD2 is involved in T-DNA integration. Although we do not currently understand the precise role that ω plays in this process, we hypothesize that this domain of VirD2, a part of the T-complex, may either be involved in establishing the correct conformation of the protein, or may directly interact with plant factor(s) that mediate T-DNA integration into the plant genome. Although we have shown evidence for the role of the ω domain of VirD2 in T-DNA integration, we cannot rule out other possible functions of the ω domain. For example, the ω domain may be important for T-complex transport before nuclear targeting. The ω domain may also play a role in stabilizing the T-complex in the nucleus and preparing it for transcription. Understanding the precise role of the ω domain warrants further investigation.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

E. coli strains were grown at 37°C on Luria-Bertani medium (Maniatis et al. 1982) and *A. tumefaciens* strains at 30°C on AB-sucrose minimal medium (Lichtenstein and Draper 1986) containing the appropriate antibiotics. Antibiotic concentrations ($\mu\text{g/ml}$) used were as follows: ampicillin, 100; kanamycin, 40 for *E. coli*; carbenicillin, 100; kanamycin, 100; spectinomycin, 50; rifampicin, 10 for *A. tumefaciens*. Table 4 lists the *A. tumefaciens* strains used in this study and their relevant characteristics. We conducted recombinant DNA experiments under P1 containment conditions as specified by the National Institutes of Health recombinant DNA guidelines.

Growth of plant cell cultures.

Tobacco (*Nicotiana tabacum*) BY-2 cells were propagated in Murashige and Skoog medium (1962; Gibco BRL, Grand Island, NY) containing 3% sucrose, 1 μg of thiamine-HCl per ml, 0.2 μg of 2,4-dichlorophenoxy acetic acid (2,4-D) per ml, and 370 μg of KH_2PO_4 per ml. *A. thaliana* Aa-0 cells were propagated in Murashige and Skoog medium containing 3% sucrose, 1 μl of vitamin stock solution per ml (0.5 mg of nicotinic acid, 0.5 mg of pyridoxine, and 0.5 mg of thiamine-HCl per ml), 100 μg of myo-inositol per ml, 0.5 mg of 2-(*N*-morpholino)ethanesulfonic acid (MES) per ml, 0.55 μg of 2,4-D per ml, and 0.8 μg of kinetin per ml. The pH of both media was adjusted to 5.7 with KOH. The cultures were shaken at 140 rpm (gyratory water bath shaker, model G76, New Brunswick Scientific, New Brunswick, NJ) at 25°C.

Infection of plant cell cultures, determination of GUS activity, isolation of plant RNA, RT-PCR, and quantitation of *gusA* mRNA.

Infection of plant cell cultures, determination of GUS activity, isolation of plant RNA, RT-PCR, and quantitation of *gusA* mRNA were performed basically as described in Narasimhulu et al. (1996). We quantitated *gusA* mRNA by adding a known quantity of in vitro-transcribed *gusA*-intron RNA to the RT-PCR reaction containing plant RNA. This control RNA was transcribed from pE1288 (Narasimhulu et al. 1996) with T7 RNA polymerase and an in vitro transcription kit (Stratagene, La Jolla, CA) according to the specifications of the supplier. We mixed various amounts of in vitro-synthesized *gusA*-intron RNA with 1 µg of plant RNA and subjected the mixture to RT-PCR as described, including the use of oligo-dT to prime cDNA synthesis. Following electrophoresis of the amplified products, we compared the signal intensity derived from the *gusA*-intron RNA (732 bp) with that derived from the processed *gusA* mRNA (543 bp). We quantitated the amount of processed *gusA* mRNA in the plant samples by comparison with the amount of *gusA*-intron RNA that yielded a signal of the same intensity.

Slight variations in some of the procedures were as follows: In the experiment examining the stability of GUS activity (Fig. 2) and in the promoter trap experiment (Table 3), 10-fold more bacteria were used to infect tobacco cells than were used by Narasimhulu et al. (1996). In addition, for these two experiments the cocultivation of the bacteria and plant cells was extended to 48 h. After 48 h, the bacteria were killed by adding either 100 µg of timentin per ml or 200 µg of cefotaxime per ml. The infected plant cells were subsequently grown for several days in the presence of these antibiotics.

In the case of the promoter trap experiments, we amplified *luc* sequences with the primers 5'-CCTCTAGAGGATGGAA CCGC-3' and 5'-CTGGCATGCGAGAATCTGACG-3'. Amplification conditions were as described in Narasimhulu et al. (1996).

DNA extraction from cell cultures and DNA blot hybridization.

Genomic DNA was extracted from rapidly growing suspension cell cultures. A standard salt precipitation method was used (Dellaporta et al. 1983). Between 1.0 and 2.0 g of cells was ground to a fine powder in a mortar in the presence of

liquid nitrogen. The powdered tissue was transferred to a capped plastic tube containing 7 ml of extraction buffer (50 mM Tris-HCl, pH 8.0; 10 mM Na₂EDTA; 100 mM NaCl; 1.0% sodium dodecyl sulfate [SDS], and 10 mM β-mercaptoethanol). The slurry was gently mixed and, after incubation for at least 5 min at 65°C, 2.5 ml of ice cold 5 M potassium acetate was added. The solution was mixed, incubated on ice for at least 15 min, and centrifuged at 8,000 × *g* at 4°C. The supernatant solution was filtered through Miracloth (Calbiochem, La Jolla, CA) into a tube containing 5 ml of isopropanol. The tubes were gently inverted several times to precipitate the DNA and were subsequently centrifuged at 8,000 × *g* to collect the precipitated nucleic acids. The pellet was rinsed with 80% ethanol, air dried, and dissolved in 500 µl of Tris-EDTA (10 mM Tris-HCl, pH 8; 1 mM Na₂EDTA). Nucleic acids were reprecipitated by the addition of 50 µl of 3 M sodium acetate and 500 µl of isopropanol. Nucleic acids were collected by a brief centrifugation, rinsed with 80% ethanol, and redissolved in 200 to 300 µl of Tris-EDTA. The DNA yield and purity were quantified by fluorometry and spectrophotometry, respectively, and the DNA integrity was evaluated by gel electrophoresis.

Five micrograms of undigested genomic DNA was size fractionated by electrophoresis through a 0.6% agarose gel. The DNA was denatured, neutralized, and transferred to a nylon membrane (Nytran; Schleicher and Schuell, Keene, NH) according to Southern (1975). The DNA was covalently cross-linked to the membrane by exposure to UV light.

The membrane was incubated for at least 4 h at 65°C in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.5% SDS, 5× Denhardt's solution (1× Denhardt's solution is 0.02% each of bovine serum albumin, ficoll, and polyvinylpyrrolidone), and 250 µg of sheared and denatured herring sperm DNA per ml. A *gusA* gene or an *A. thaliana* PAL (phenylalanine-ammonia lyase) gene radiolabeled with ³²P-dCTP (Multiprime DNA Labelling System; Amersham Life Science, Arlington Heights, IL) was added and incubated with the membrane for at least 24 h at 65°C. The membrane was washed 4 times with 0.3× SSC, 1.0% SDS for at least 1 h each time at 65°C, and exposed to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA) for approximately 24 h. Similar conditions and procedures were used for DNA blot transfer of the RT-PCR product and hybridization with the *luc* gene.

Table 4. *Agrobacterium tumefaciens* strains used in this study

Strain	Description	Antibiotic resistance ^a	Reference
At746 (WR1826) ^b	<i>virD2</i> ω substitution in WR1715 ^c	Rif, Cb, Kan	Shurvinton et al. 1992
At747 (WR1753) ^b	Wild-type <i>virD1+virD2</i> in WR1715 ^c	Rif, Cb, Kan	Shurvinton et al. 1992
At749 (WR1749) ^b	Δ <i>NruI virD2</i> in WR1715c	Rif, Cb, Kan	Shurvinton et al. 1992
At751 (WR1766) ^b	Δ <i>NLS virD2</i> in WR1715c	Rif, Cb, Kan	Shurvinton et al. 1992
At822	pBISN2 in At747	Rif, Cb, Kan, Spc	This study
At823	pBISN2 in At749	Rif, Cb, Kan, Spc	This study
At827	pBISN2 in At751	Rif, Cb, Kan, Spc	This study
At829	pBISN2 in At746	Rif, Cb, Kan, Spc	This study
At894	pBISN2 in A136 ^d	Rif, Cb, Kan, Spc	This study
At898	pKM1 in At746	Rif, Cb, Kan, Spc	This study
At899	pKM1 in At747	Rif, Cb, Kan, Spc	This study

^a Rif, rifampicin; Kan, kanamycin; Cb, carbencillin; Spc, spectinomycin.

^b Numbers within parentheses are Ream laboratory strain numbers.

^c WR1715 is an octopine-type *A. tumefaciens* strain harboring a 295 codon deletion in *virD2* (Shurvinton et al. 1992).

^d A136 is a C58 strain lacking a tumor-inducing (Ti) plasmid.

Construction of the promoter trap vector.

The plasmid pTDC1 (Filichkin and Gelvin 1993), containing the left and right T-DNA borders from the T_R T-DNA of pTiB₆806 and an *Xba*I site between the borders, was digested with *Xba*I and the overhanging ends filled in with the Klenow fragment of DNA polymerase I and nucleotide triphosphates. We digested the plasmid pCNL65 (Liu et al. 1992) with *Hind*III and *Nco*I to release a promoterless *gusA*-intron gene plus an octopine synthase polyadenylation signal sequence, and filled in the overhanging ends with the Klenow fragment of DNA polymerase I and nucleotide triphosphates. This promoterless *gusA*-intron gene was cloned into the *Xba*I-digested and blunted plasmid pTDC1. This resulting plasmid (pE1323) was digested with *Bst*EII (which cuts after the polyadenylation signal sequence) and *Bgl*II linkers were ligated to this site, resulting in the plasmid pE1341.

A *luc*-intron gene, originally contained in the plasmid pCAL56 (obtained from Peter Christie, University of Texas Health Sciences Center, Houston) was digested with *Bam*HI and *Bgl*II to release the *luc*-intron gene with a CaMV 35S promoter and a nopaline synthase polyadenylation signal sequence. This fragment was cloned into the *Bgl*II site of pE1341, resulting in the plasmid pE1349. This plasmid was digested to completion with *Sma*I and partially with *Pst*I to release the newly constructed T-DNA region. The *Sma*I-*Pst*I fragment was cloned into the plasmid pUCD2 (Close et al. 1984) that was previously digested with *Eco*RV and *Pst*I, resulting in pKM1. pKM1 was mobilized into the appropriate *A. tumefaciens* strains by triparental mating (Ditta et al. 1980).

Tumor DNA extraction.

Plant tumor DNA was extracted by grinding 7 to 8 g of tumor tissue in the presence of liquid nitrogen and isolation buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM Na₂EDTA, and 1.0% SDS) in a mortar. The extract was centrifuged for 10 min at 9,000 rpm (Sorvall RC-5B refrigerated superspeed centrifuge, E. I. Du Pont de Nemours, Wilmington, DE) at 4°C. The supernatant solution was extracted twice with phenol/chloroform (1:1). DNA was precipitated from the supernatant fluid by adding 1/10 vol of 3 M sodium acetate and 2.5 vol of ethanol. The DNA was wound on a sterile glass rod, air dried, and dissolved in sterile H₂O. This DNA solution was treated with RNase (100 µg/ml) at 37°C for 20 min, and purified by passing through a QIAGEN 500 column (Qiagen, Chatsworth, CA). The DNA yield and purity were quantitated by fluorometry and spectrophotometry, respectively, and the DNA integrity was evaluated by agarose gel electrophoresis.

PCR for detection of T-DNA genes in transgenic tobacco calli.

The PCR reaction was performed in a total volume of 25 µl. The mixture contained 50 ng of plant tumor DNA, 1× PCR reaction buffer (Promega, Madison, WI), 0.25 mM dNTPs, 25 pmol of each of the appropriate primers, and 1 U of *Taq* DNA polymerase (Promega). The reaction was performed in a PTC-150 minicycler (MJ Research, Watertown, MA) as follows. For the *picA* primers (Yusibov et al. 1994), the amplification was conducted with the following program: 94°C for 3 min, 56°C for 5 min, and 72°C for 2 min,

as a first cycle; 94°C for 20 s, 56°C for 1.5 min, and 72°C for 2 min, for 35 cycles; 94°C for 1 min, 56°C for 3 min, and 72°C for 5 min in the final cycle. For the *tms* primers (Yusibov et al. 1994) and *rb* (right border) primers, the amplification was conducted with the following program: 94°C for 5 min, 63°C for 2 min, and 72°C for 1 min, as a first cycle; 94°C for 30 s, 63°C for 1.5 min, and 72°C for 1.5 min, for 30 cycles; 94°C for 1 min, 56°C for 3 min, and 72°C for 5 min in the final cycle. The sequences of the *rb* primers were designed according to the T-DNA sequence of Barker et al. (1983; nucleotide positions in this sequence are indicated by [#]):

rb1, 5'-CCAGTCAGCATCATCACACC-3' [#14,161-14,141];
rb2, 5'-CTCGCAATTGAGGTCTACAGG-3' [#14,007-13,987];
rb3, 5'-GATCCATCTTGAGACCACAGG-3' [#13,911-13,891];
rb4, 5'-CGTAAGCGCTTACGTACATGG-3' [#13,850-13,830];
rb5, 5'-CTCATCCACATTGATGGTAGG-3' [#13,760-13,740];
rb6, 5'-GAGTTGACGCAACTTCGAACG-3' [#13,152-13,172].

The amplified DNA products from the *picA* primers were analyzed on a 1% agarose gel, whereas the amplified products from the *tms* and the *rb* primers were analyzed on 1.2% agarose gels.

In vitro cleavage of right border of T-DNA by VirD2.

The wild-type VirD2 and the ω deletion mutant VirD2 (lacking the C-terminal six amino acids) were cloned into the plasmid pET23a (Novagen, Madison, WI). To make these constructions, a *Nde*I site (including the translation start codon ATG) was affixed to the 5' end of the *virD2* gene with the PCR, and the resulting 379-bp *Nde*I-*Sal*I fragment of *virD2* (encoding the N-terminal portion of VirD2 protein) was cloned into the *Nde*I and *Sal*I sites of pET23a. The wild-type *virD2* gene was reconstituted by linking a 1,110-bp *Sal*I fragment encoding the C-terminal portion of VirD2 protein to the construction described above. The ω -deletion *virD2* gene was constructed by replacement of the wild-type C-terminal *Sal*I fragment with a *Sal*I fragment, generated with the PCR, that deleted the C-terminal six amino acids from VirD2. All constructions were confirmed by DNA sequence analysis. VirD2 protein was expressed in *E. coli*, and purified as described by Jasper et al. (1994). A 498-bp *Rsa*I fragment containing the octopine-type T_R right border sequence (nucleotides 23,715 to 24,213; Barker et al. 1983) was cloned into the *Hinc*II site of pUC118 and used as a target site for the VirD2 endonuclease. This plasmid was first digested with *Eco*RI prior to the preparation of a uniformly labeled, single-stranded DNA fragment containing the T-DNA border by one-directional PCR with the primer 5'-CCCAGTCACGACGTTGTAAACGACGGCCA GTGCC-3' in the presence of [α]³²P-dCTP. The PCR was carried out for 6 cycles with the following program: 94°C, 5 min; 56°C, 5 min; 72°C, 10 min. In vitro cleavage of the resulting labeled, single-stranded DNA was performed as described (Pansegrau et al. 1993). The labeled, single-stranded DNA fragment was incubated with purified wild-type VirD2 or the ω mutant VirD2 at 37°C in 20 µl of reaction buffer (20 mM Tris-HCl, pH 8.5, 5 mM MgCl₂, 50 mM NaCl) for 30 min. The reaction products were separated by electrophoresis through 4% polyacrylamide gels containing 8 M urea in TBE (Tris-borate-EDTA) buffer (Maniatis et al. 1982). The gels were vacuum dried and exposed to X-ray film.

Construction of *gusA-virD2* gene fusion plasmids.

A 770-bp *KpnI-EcoRI* fragment from the *virD2* gene of the octopine-type Ti plasmid pTiA6 contains the C-terminal 37 amino acids, including the bipartite NLS and the ω regions. This fragment was cloned into the *KpnI* and *EcoRI* sites of pUC19, generating the plasmid pE1299. The plasmid pRTL2-GUS/N1a (Restrepo et al. 1990), containing a CaMV 35S promoter, the tobacco etch virus (TEV) leader, a *gusA* gene, the TEV N1a NLS region, and a CaMV 35S polyA signal sequence, was digested with *XbaI*. The *XbaI* site was made blunt with the Klenow fragment of DNA polymerase I and dNTPs. The resulting plasmid was digested with *BglII*, deleting the TEV N1a NLS region. Into this linearized molecule was cloned a *BamHI-HincII* fragment of pE1299. The resulting plasmid, pE1300, contains an in-frame *gusA-virD2* NLS fusion gene under the transcriptional control of the CaMV 35S promoter. To mutagenize the *virD2* region, we cloned a *PstI* fragment from pE1300, containing the *virD2* region, into the *PstI* site of pBluescript KS⁻, generating the plasmid pE1303 (GUS-VirD2-NLS). An *SmaI* fragment from pE1303 was cloned into the *SmaI* site of pBluescript KS⁻. Oligonucleotide-directed mutagenesis of *virD2* was performed on this *SmaI* fragment as described by Kunkel et al. (1987), with the following primers:

mut.NLS1, 5'-CAACCGGAAGCTT CTTGGAAGTTTAAAAAGGACCG TCACGATGGA-3';

mut.NLS2, 5'-GATGGAGAATTGGGTTGGAAGAAATTTAAAAAGGG TAATCGTCGCGAC-3';

mut.spacer, 5'-CCGCGTGACCGTCACAACCTGGCAGCTGGGTGGACG CAAACG-3';

$\Delta\omega$, 5'-GGTAATCGTCGCGACACCTAGATCTAGGAAGGACCGAATA-3';

mut.total NLS, 5'-CAACCGGAAGCTTATGCAGAGCGTCTGCATGTCC GTCTCCGTTTAAAGAGAGGGTACGCGAACGTACAAGAGG-3'.

To generate mut.NLS 1+2, we used the mut.NLS2 oligonucleotide to mutagenize further a derivative of pE1303 that contained the mut.NLS1 mutation. The mutant *SmaI* fragments were cloned back into pE1303, replacing the wild-type sequence. The sequences of the mutant *gusA-virD2* fusion genes were confirmed at the Purdue University DNA sequencing center.

Protoplast preparation, electroporation, and histochemical GUS assay.

Tobacco BY-2 suspension cells were harvested by centrifugation (50 ml, 5 min at 1,000 rpm [Sorvall GLC-2 centrifuge, DuPont Instruments, Newtown, CT]) and resuspended in an equal volume of protoplast isolation solution (0.25 M mannitol, 12 mM sodium acetate, and 66 mM CaCl₂, pH 5.7) containing 1.0% cellulase R10 (Onozuka, Tokyo), 0.5% hemicellulase (Sigma, St. Louis, MO), and 0.1% Y-23 pectolyase (Seishin, Tokyo). The cells were incubated for 4 h at room temperature with gentle shaking (40 rpm [Orbit Environ-Shaker, Lab-Line Instruments, Melrose Park, IL]). Protoplasts were washed twice with protoplast isolation solution and purified by centrifugation (900 rpm, 5 min [Sorvall GLC-2 centrifuge, DuPont Instruments, Newtown, CT]) through floating solution (0.3 M sucrose, 4.3 g of Murashige and Skoog salts per liter, 1 mg of thiamine per liter, 370 mg of KH₂PO₄ per liter, 99 mg of myo-inositol per liter, 2.88 g of L-proline per

liter, 100 mg of enzymatic casein hydrolysate per liter, 97.6 mg of MES [2-(*N*-morpholino)ethanesulfonic acid] per liter, pH 5.7). Protoplasts formed a band at the top of the gradient and were removed and washed with electroporation solution (10 mM HEPES [N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.2, 10 mM NaCl, 4 mM CaCl₂, 120 mM KCl, and 0.2 M mannitol). The protoplast concentration was counted in a hemacytometer and adjusted to 3×10^6 protoplasts per ml. Protoplasts were heated for 5 min at 42°C, then placed on ice for 10 min. For electroporation, 10^6 protoplasts (300 μ l) and 50 μ g of DNA were mixed in electroporation cuvettes and placed on ice for 10 min. The protoplasts were electroporated for 30 ms at 130 V with a Gene Pulser electroporation apparatus (BioRad, Hercules, CA). The protoplasts were left on ice for 10 min after electroporation, then diluted to 3.5×10^5 protoplasts per ml in 3.5 ml of suspension medium (BY-2 cell propagation media containing 0.4 M mannitol) and incubated for 20 h at 24°C.

Cells were assayed for GUS activity as described previously (Jefferson 1987; Gallie et al. 1988). Two hundred and fifty microliters of protoplasts was combined with 2 ml of GUS assay solution containing 2 mM X-gluc and the assay was performed as described by Howard et al. (1992). Blue color usually began to appear after 20 min of incubation. Protoplasts were observed and photographed after 2 h with a Diaphot-TMD inverted phase contrast microscope (Nikon, Tokyo). Each experiment was conducted a minimum of four times.

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