The Omega Sequence of VirD2 Is Important but Not Essential for Efficient Transfer of T-DNA by *Agrobacterium tumefaciens*

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The VirD2 protein of *Agrobacterium tumefaciens* contains defined sequences necessary for processing and transferring the T-DNA during transformation of plant cells. We performed a mutational analysis of the conserved omega sequence of VirD2, whose role has proven to be difficult to elucidate so far. In this report, we show that a deletion of these 5 amino acids or their replacement by 5 glycines reduced T-DNA transfer considerably, compared with wild type, demonstrating that the omega sequence is important for the efficient transfer of T-DNAs. However, the efficiency and pattern of integration of the T-DNAs were not affected by any modifications of the omega sequence. The importance of the C terminus of VirD2 for T-DNA transfer is discussed.

Agrobacterium tumefaciens is a bacterium that evolved to transfer part of its tumor-inducing (Ti) plasmid into the nuclear genome of plants, where it gets integrated. This sequence is called the T-DNA and it is delimited by two 25-bp border sites, called the right and left border. The production and transfer of the T-strand, a single-stranded version of the T-DNA, are mediated by bacterial virulence proteins (Vir proteins; reviewed by Ream 1989; Zambryski 1992; Hooykaas and Beijersbergen 1994; Tinland 1996).

Mutational analysis has demonstrated that VirD2 plays various important roles during the process of T-DNA transformation. Initiation of processing at the right border is performed by VirD2, which, together with VirD1, nicks the bottom strand and subsequently binds covalently to the 5' end of the single-stranded T-DNA. While the first 262 N-terminal amino acids (out of a total of 424 amino acids) of VirD2 are sufficient for T-strand production (Ward and Barnes 1988), full virulence requires the presence of the C-terminal half as well (Stachel et al. 1987; Steck et al. 1990) (Fig. 1A). Sequence comparison has shown that the first 203 amino acids are 90% conserved among VirD2 proteins identified in various

Agrobacterium strains (Steck et al. 1990; Wang et al. 1990), while the other 221 amino acids share only 25% homology. Interestingly, within this poorly conserved part of the protein, two sequences at the very C terminus have been conserved in all VirD2 proteins analyzed. The first element is the nuclear localization sequence (NLS), which enables VirD2 to pilot the attached T-DNA into the nucleus (Howard et al. 1992; Shurvington et al. 1992; Tinland et al. 1992; Rossi et al. 1993b). The 5 amino acids DGRGG adjacent to the NLS, termed the omega sequence (Fig. 1), have been implicated in tumorigenesis (Shurvington et al. 1992).

In this report, we used strains modified in the omega sequence to measure the various steps of T-DNA-mediated transformation and hence identify the level at which omega is important. We constructed a *virD2* gene lacking only the omega sequence and used it to complement an *Agrobacterium* strain containing a complete and precise deletion of the *virD2* gene. Our results show that omega and, in more general terms, the C-terminal end, are required by VirD2 to carry out efficient T-DNA transfer.

RESULTS AND DISCUSSION

Two mutations were introduced into VirD2, at the position of the omega sequence, in order to analyze its possible function in T-DNA transformation. The mutation VirD2 $\Delta\omega$ carried a complete deletion of the 5 highly conserved amino acids (plasmid pAvirD2 $\Delta\omega$; Fig. 1B) and was designed to test whether or not the amino-acid sequence of omega plays an active role in transformation. In mutation VirD2 ω /G, amino acids D419 and R421 were replaced by glycines, thereby exchanging omega with a pentaglycine stretch (plasmid pAvirD2 ω /G), enabling us to study the effect of a neutral sequence on the functions of VirD2 (Fig. 1B). A *virD2*-deficient strain, AT Δ virD2, which is precisely deleted in the *virD2* open reading frame, was constructed (Table 1; Figs. 2 and 3). Mutations *virD2* $\Delta\omega$ and *virD2* ω /G as well as the intact *virD2* gene were introduced into this *virD2*-deficient strain AT Δ virD2 (see Materials and Methods), resulting in the strains AT*virD2* $\Delta\omega$, AT*virD2* ω /G, and AT*virD2*⁺, respectively. The binary plasmid pTd33, carrying a T-DNA with the *uidA* gene encoding the β -glucuronidase protein, together with the *nptII* gene, which provides kanamycin resistance as a selectable marker (Fig. 4A), were introduced into these strains. The resulting bacteria were tested for formation of T-strands,

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their transfer into the plant cell nucleus, and their integration into the plant genome.

T-DNA processing.

VirD2, in association with VirD1, cleaves the T-DNA borders during processing in the bacterium. We measured the ability of the omega mutant strains AT*virD2*Δ ω and AT-*virD2*Δ ω /G to nick the right border of the binary vector pTd33 (Fig. 4A). Total DNA from induced *Agrobacterium* cells was cut with *Hind*III, which generates a T-strand reduced in size from 6 to 3 kb if the right border has been processed. Detection of these processed products was performed by hybridizing a *uidA*-specific probe to a nondenaturing Southern blot (see Materials and Methods). The omega mutants were all capable of nicking the T-DNAs at the right border with efficiencies similar to that of the AT*virD2*⁺ strain (Fig. 4B).

Fig. 1. A, Schematic representation of the VirD2 protein. I, II, III: motifs common with the bacterial conjugation TraI protein of the plasmid RP4. NLS: nuclear localization sequences. ω : Omega (DGRGG). Shaded region represents the conserved part of VirD2. B, Omega sequence of wild-type VirD2 (in bold) encoded by octopine pTiA6NC (used in this work), nopaline pTiC58, and *Agrobacterium rhizogenes* pRiA4b plasmids (Wang et al. 1990). The mutant VirD2Δ ω has a deletion of the 5 conserved amino acids of omega (replaced by five dashes) and VirD2Δ ω /G has two point mutations (underlined glycines) that convert omega into a pentaglycine sequence. Amino acids in italics belong to the NLS of VirD2. An asterisk indicates the C terminus. C, Schematic representation of the strategy used to construct the omega mutations of *virD2*. E: *Eco*RI; B: *Bam*HI. pVD43 contains the *vir* promoter and *virD2* gene. Primers pr ω 1 or pr ω 2 were used together with pr5 in a polymerase chain reaction to amplify the complete plasmid. After sequencing, the *Bam*HI-*Eco*RI fragment containing the mutation was used to replace the corresponding fragment from the wild-type *virD2* sequence of plasmid pAVD43, resulting in the mutants p*AvirD2*Δ ω and p*AvirD2*Δ ω /G.

T-DNA transfer efficiency.

T-DNA transfer was tested after co-cultivation of tobacco seedlings with the different *Agrobacterium* strains. The presence of the *uidA* and *nptII* genes on the T-DNA of pTd33 allowed us to measure transient expression and stable integration of T-DNA-encoded genes within the same batch of inoculated seedlings (Fig. 4A; see Materials and Methods). To optimize the sensitivity of the transfer assay and be able to count single transfer events, the strains to be tested have been diluted against the transfer-deficient strain ATΔ*virD2*. This choice allows maintaining the overall concentration of bacteria in the inoculum constant in the different co-cultivations.

We defined the relative transfer efficiency as the number of T-DNA molecules transferred to the plant cell nucleus, by the bacterial strain being tested, divided by the number obtained from wild-type bacteria. This was measured by the activity of β -glucuronidase (number of blue spots per tested seedling) resulting from the transient expression of the transferred *uidA* gene. The strain AT*virD2*⁺(pTd33) was found to have a transfer efficiency comparable to that of the original wild-type strain GV3101(pPM6000; pTd33) carrying an endogenous *virD2* gene, which confirmed previous observations (Rossi et al. 1993b).

Table 2 shows the results obtained from three independent experiments. The omega-deleted strain AT*virD2*Δ ω (pTd33) showed a marked decrease in transfer, giving only a third of the efficiency of the wild-type strain AT*virD2*⁺(pTd33). This indicated that the omega sequence is important, but not essential, for any of the steps prior to (and including) nuclear entry and double-stranded DNA formation. Omega and the NLS are the only conserved sequences in the otherwise nonconserved second half of the VirD2 protein (Fig. 1A), which indicates an important function for the C terminus of VirD2. The importance of omega may be more significant in nature, where the infection efficiency of *Agrobacterium* is a key factor in the progression of its life cycle.

The importance of the omega domain could be at the level of its amino acid sequence, as a recognition signal, or at the level of its influence on the conformation of the protein. The fact that omega can be deleted without abolishing transfer shows that its importance is not as a recognition signal per se. In order to evaluate whether an omega-containing peptide would interfere with transfer mediated by VirD2, we measured T-DNA transfer performed either by the wild-type VirD2, or by one of the omega mutants, from a strain carrying an internal deletion of *virD2* that retained the omega sequence (data not shown). Since transfer efficiencies were not affected, this omega-containing peptide did not compete with the wild-type VirD2 protein for a particular recognition site in the bacterium. Furthermore, this omega peptide could not complement the two omega mutations in *trans* (A. M. Bravo-Angel, unpublished results). Thus, modifications in a local region at the C terminus apparently affect the structure and consequently a particular function of VirD2 (see below).

Surprisingly, the glycine replacement mutant AT-*virD2*Δ ω /G(pTd33) showed a transfer efficiency of 6% relative to the wild-type strain AT*virD2*⁺(pTd33) (Table 2). The low transfer phenotype observed may indicate that the glycine mutation had a more severe impact on the same step in transfer than the deletion mutation did. Alternatively, the pentaglycine might change the C terminus into a different conforma-

tion that could affect transfer at several levels. Because glycines can increase the flexibility of a protein, it is possible that this glycine-rich C terminus can interfere with interactions between particular domains of VirD2 or between VirD2 and other proteins. One could also imagine that the glycines influence the correct local folding of the C terminus. Alternatively, or in addition, the adjacent NLS sequence may be affected in its accessibility to the nuclear transport machinery. It has been described that the NLS of the oncoprotein c-Myc can be influenced by its adjacent amino acid environment (Makkerh et al. 1996). We also cannot exclude the possibility that the differences in the charge distribution of the C terminus have an influence on the properties of VirD2.

The C-terminal region of the protein has been previously reported to be very sensitive to alterations. It was found that a nopaline strain VirD2 protein, devoid of its two C-terminal arginines, could only partially complement a *virD2*-deficient strain (Steck et al. 1990). Furthermore, this C-terminal region of the protein showed an unexpected sensitivity when assayed for the NLS sequences located within this region (Rossi et al. 1993b).

Interestingly, the nonconserved region separating the functional domains of VirD2 can be deleted by 70 amino acids (from

the *Bam*HI site to the NLS sequence; see Figure 1A) without affecting any step of transformation (Koukolíková-Nicola et al. 1993; B. Tinland, *unpublished results*). This region might provide more flexibility within the protein or it might only be a remnant from an “earlier” version of this protein.

Integration efficiency.

We proceeded to analyze whether a step downstream from nuclear entry was perturbed by the changes in the omega sequence. By exploiting the *nptII* gene located adjacent to the *uidA* gene on the T-DNA of pTd33 (Fig. 4A), we estimated the efficiency of T-DNA integration into the plant chromosomal DNA after selection for kanamycin-resistant calli. The transformation efficiency of a strain corresponds to the number of kanamycin-resistant calli per seedling, compared with the number produced by a wild-type bacterium. We thus define the integration efficiency as the ratio of transformation efficiency to transfer efficiency (as defined above). This value represents the fraction of T-DNA molecules that integrate out of those that entered the nucleus. Experimentally, we define the integration efficiency as the number of kanamycin-resistant calli divided by the number of blue spots. The integration efficiency of mutant strain AT*virD2*Δω(pTd33) was comparable to that of the wild-type

Table 1. Bacterial strains and plasmids

Name	Relevant characteristics	References
<i>Agrobacterium tumefaciens</i> strains		
GV3101(pPM6000)	pPM6000 is a disarmed Ti plasmid of a wild-type strain; rifampicin resistant	Bonnard et al. 1989
ATΔ <i>virD2</i>	GV3101(pPM6000) carrying a total deletion in the <i>virD2</i> gene	This work
AT <i>virD2</i> ⁺	ATΔ <i>virD2</i> , complemented by the wild-type VirD2	This work
AT <i>virD2</i> Δω	ATΔ <i>virD2</i> , complemented by the omega deletion mutant of VirD2	This work
AT <i>virD2</i> Δω/G	ATΔ <i>virD2</i> , complemented by the omega glycine replacement mutant of VirD2	This work
<i>Escherichia coli</i> strains		
NM522	Strain used for cloning	Gough and Murray 1983
S17-1	Helper strain used for conjugation	Simon et al. 1983
Plasmid		
pARO-181	pUC derivative with the origin of transfer of plasmid RP4, does not replicate in <i>Agrobacterium</i>	Parke 1990
pCVK225	Cosmid containing the <i>virC</i> , D and E operons of plasmid pTiA6	Stachel and Nester 1986
pVD43	<i>virD</i> promoter and <i>virD2</i> in pUC18	Rossi et al. 1993b
pAVD43	<i>virD</i> promoter and <i>virD2</i> in pARO-181	Rossi et al. 1993b
pAvirD2Dw	<i>virD</i> promoter and <i>virD2</i> omega total deletion in pARO-181	This work
pAvirD2w/G	<i>virD</i> promoter and <i>virD2</i> omega glycine replacement in pARO-181	This work
pTd33	Binary plasmid containing an <i>uidA</i> gene and an <i>nptII</i> gene; gentamycin resistant	Tinland et al. 1995

Name Sequence

Construction of the *virD2* deletion strain

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pr1  TGACTTAAAGCTTTTGGGATGGTGGATCTCGG
pr2  TGATTCATTAAGCTTGTTGATATCAATTTTCTCCCGTCAGGTGCGTG
pr3  CAATGACAAAAGCTTTAACCCGGGAAATGGTCAGTTCACGATACGCTC
pr4  GCGCAAAAAGCTTTATACTTTTCGCC
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Construction of the omega mutants

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pr5  ATCTAGCAGATCTTTGAATTCGTAATCATGGTCATAGCTGTTTCCTG
prw1 ATCTAGCAGATCTTTGAATTCATTTCGGTCCTTCCTGTCTCTAGGTGTCGCGACGATTACCTC
      TTGCACGTTTGCG
prw2 ATCTAGCAGATCTTTGAATTCATTTCGGTCCTTCCTGTCTCTAGGTCCCCCGCCCCACCGT
      CGCGACGATTACCTCTTGAC
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Fig. 2. Sequences of the oligonucleotides used. The underlined sequences AAGCTT, GATATC, CCCGGG, AGATCT, and GAATTC correspond to the restriction sites for *Hind*III, *Eco*RV, *Sma*I, *Bgl*II, and *Eco*RI, respectively.

strain AT*virD2*⁺(pTd33), although a slight increase was observed. This indicates that omega sequence was not required for efficient integration (Table 2). Interestingly, strain AT*virD2*Δω/G(pTd33) showed a slightly stronger but reproducible increase in integration efficiency, of a factor of about 1.6, when compared with the wild-type strain, suggesting that in replacing omega, the glycines could contribute to a more efficient integration. Further work is required to evaluate the significance and to explain this result.

Patterns of the integrated T-DNAs.

Although the omega sequence did not affect the efficiency of integration, it could be important for the preservation of

Fig. 3. Construction of a Ti plasmid with a deletion of *virD2*. Restriction sites are indicated as follows: H, *Hind*III; S, *Sma*I; RV, *Eco*RV. Arrows marked pr1, pr2, pr3, and pr4 indicate the primers used for polymerase chain reaction to create a *virD* operon lacking the *virD2* gene. The resulting fragments were then separately inserted as *Hind*III fragments, into pUC18. An *Sma*I fragment from pRD2, containing the insert, was then inserted into the *Eco*RV site of pLD2, resulting in pΔ*virD2*. After transfer to pARO-181, resulting in pΔΔ*virD2*, the construct was introduced into *Agrobacterium tumefaciens* GV3101(pPM6000) where, by homologous recombination, the fragment replaced the endogenous counterpart, resulting in a *virD2*-deleted strain, ATΔ*virD2* (see Materials and Methods).

the right border of the T-DNA during the integration process. T-DNAs transferred by a previously described mutation of *VirD2* could integrate into the genome efficiently, but the integrated copies were truncated at their 5' end, while those transferred by a wild-type *VirD2* were conserved in six out of eight cases analyzed (Tinland et al. 1995). The patterns of the integrated T-DNAs resulting from transfer by the mutant strains AT*virD2*Δω(pTd33) and AT*virD2*Δω/G(pTd33) were analyzed by digestion of DNA extracted from transformed regenerants with the restriction enzymes *Hind*III and *Eco*RI followed by Southern blots with *uidA* as a probe (see Materials and Methods). Figure 5 shows the results obtained for the mutant strain AT*virD2*Δω/G(pTd33). Digestion with *Hind*III revealed that the transformants had single inserts, except number 7, which contained two T-DNAs. The *Eco*RI site at 7 bp from the right border had been designed as a marker for the conservation of the 5' end of the integrated T-DNA (Tinland et al. 1995). With a specific probe for the *uidA* gene of the T-DNA (Fig. 4A), it was shown that although transfer efficiencies for mutant strain AT*virD2*Δω/G(pTd33) were reduced to about 5% (see above), the right borders of the T-DNAs were conserved in six out of seven of the cases, comparable to what was observed for wild-type *VirD2* (lanes 1 and 9). Transformant 4 was truncated at the 5' end, and had lost the diagnostic *Eco*RI site. Also, one of the inserts of transformant 7 is truncated. Similar conserved patterns were observed for strain AT*virD2*Δω(pTd33) (data not shown), allowing us to conclude

Fig. 4. A, T-DNA region of binary plasmid pTd33. The T-DNA is delimited by the flags representing the two borders. P and t are the 35S promoter from cauliflower mosaic virus and the terminators of the neomycin phosphotransferase (kanamycin resistance) and *uidA* for β-glucuronidase. Line under the T-DNA represents the region used to probe the T-DNA. H, E, and X are restriction sites *Hind*III, *Eco*RI, and *Xba*I, respectively, used to analyze T-DNA integration patterns. B, Nondenaturing Southern blot of total DNA from induced *Agrobacterium* strains ATΔ*virD2*, AT*virD2*⁺, AT*virD2*Δω, and AT*virD2*Δω/G. The DNA was digested with *Hind*III, revealing processing of T-strands at the right border. The probe used was an internal fragment of the *uidA* gene (see above).

that the omega sequence was not essential for integration and was therefore not involved in any particular interaction of the T-complex with the integration machinery. Moreover, the pentaglycine motif did not interfere with this process either.

The proteins involved in the process of *Agrobacterium*-mediated transformation have long been sought, and have been expected to be of both bacterial and plant origin. The current model states that the major roles of the bacterial proteins accompanying the T-DNA to the plant cell consist of transporting the molecule efficiently and as intact as possible, into the nucleus (Zambryski 1992; Tinland 1996). Our results reinforce the model that proposes that the host cell determines the efficiency and mode of integration (Bundock et al. 1995; Sonti et al. 1995; Tinland et al. 1995), and obviates the need for omega in this step, as was suggested (Narasimhulu et al. 1996). The data indicate that omega itself does not play an essential role in T-DNA transfer, since, in its absence, all the processes leading to integration can still be performed with an efficiency of above 30% of the wild-type VirD2. Nevertheless, it is clear that the C-terminal region plays an important role in the transfer process, a role that is inhibited by certain modifications like the addition of glycines or other amino acids (Shurvington et al. 1992; S. Gelvin, *personal communication*). We can exclude a specific role during processing or T-DNA integration. It is probable that it is the piloting function of VirD2, either from the bacterium to the plant cell, or from the plant cell cytoplasm to the nucleus, that requires a correct C terminus.

MATERIALS AND METHODS

Strains, plasmids, and media.

The strains and plasmids used are listed in Table 1; bacterial and plant media were prepared as described (Murashige and Skoog 1962; Sambrook et al. 1989).

Construction of an *Agrobacterium* strain lacking the gene *virD2* (Fig. 3).

An *Agrobacterium* strain completely devoid of *virD2* was constructed. First, the two regions flanking *virD2* were cloned from the cosmid pVK225 (Stachel and Nester 1986). The sequences of *virC1-virC2-virD1* and *virD3-virD4* were amplified by polymerase chain reaction, with the set of primers pr1-pr2 and pr3-pr4, respectively (Fig. 2). The resulting fragments were inserted as *Hind*III fragments into pUC18 to give the plasmids pLD2 and pRD2. An *Sma*I fragment of pRD2, containing the *virD3-virD4* sequence, was then subcloned into the unique *Eco*RV site of pLD2, to yield the plasmid pΔ*virD2*. After sequencing, the fragment containing *virC2,C1,D1,D3,D4* was cloned into the mobilizable, kanamycin-resistant vector pARO-181 (Parke 1990) as a 3-kb *Hind*III fragment and was introduced into the *Escherichia coli* helper strain S17-1 for mating with the wild-type *Agrobacterium* strain GV3101(pPM6000). The following steps were as previously described (Rossi et al. 1993b). Because the transferred pARO vector pΔ*virD2* is unable to replicate in *Agrobacterium*, its integration by homologous recombination into the pTi plasmid

Table 2. Efficiency of transfer, transformation, and integration of *Agrobacterium* strains containing the wild-type and omega mutants of *virD2*

No. ^a	VirD2 allele	Dilution ^b	Total spots/ total seedlings	Transfer efficiency spots/ seedling × dilution factor ^c	Relative transfer efficiency (%) ^d	Total calli/total seedlings	Transformation efficiency calli/seedling × dilution factor	Relative transformation efficiency (%) ^d	Integration efficiency transformation/transfer efficiencies	Relative integration efficiency (%) ^e
1	wt	1/100	178/75	237	100	(154/303) ^f	(51)	100	(0.21)	100
		1/500	50/91	275		75/286	131		0.51	
	Δω	1/100	54/75	72	27	(111/357) ^f	(31)	38	(0.43)	141
		1/500	11/82	67		28/280	50		0.72	
	ω/G	1/50	18/86	10.5	4	47/309	7.6	6	(0.71)	151
		1/100	8/90	9		26/356	7.3		0.77	
2	wt	1/100	138/30	460	100	(83/184) ^f	(45)	100	(0.10)	100
		1/1,000	14/27	519		12/216	55		0.11	
	Δω	1/50	126/33	191	42	(63/152) ^f	(21)	51	(0.11)	127
		1/100	70/32	219		66/234	28		0.14	
	ω/G	1/50	21/27	39	9	39/239	8	15	(0.20)	172
		1/100	15/32	47		24/286	8		0.19	
3	wt	1/500	62/29	1,070	100	52/231	113	100	0.10	100
		1/1,000	39/35	1,110		NT ^g			NA ^g	
	Δω	1/100	63/29	217	21	78/273	29	29	(0.13)	140
		1/500	15/30	250		16/216	37		0.14	
	ω/G	1/50	40/27	74	6	59/242	12	10	(0.16)	170
		1/100	17/26	65		28/252	11		0.17	

^a Refers to three experimental series.

^b 1/50, 1/100, 1/500, 1/1000 correspond to the ratio of the strain to be tested to the transfer deficient strain ATΔ*virD2*(pTd33) used to inoculate the seedlings.

^c Transfer and transformation efficiencies of the strains.

^d Relative transfer and transformation efficiencies are average values calculated from the two dilution values, compared with the wild-type (wt) strain.

^e Relative integration efficiency was calculated with only the values from the highest dilution tested.

^f When the number of calli per seedling is too high (roughly >0.3), the calculation of the transformation efficiency is no longer accurate, which leads to an underestimation of the transformation efficiency because not all the transformation events can be monitored (one seedling can reveal only one integration event). In such cases, only the value corresponding to the higher dilution is used to calculate the relative integration efficiency and the other value is placed in parentheses (the transfer efficiency is independent of the dilution factor because several transfer events can be monitored per seedling).

^g NT: not tested; NA: not applicable.

can be screened for by selecting the bacteria for kanamycin resistance. Selected colonies were analyzed by Southern blot to confirm that the first recombination had taken place correctly. Bacteria containing the correctly integrated pAΔ*virD2* vector were grown overnight in the absence of the antibiotic and plated to single colonies. These were screened for the loss of kanamycin resistance by replica plating, indicative of a second recombination event. Discrimination between recombinants in which the wild-type sequence was restored and those carrying the incoming fragment lacking the *virD2* gene was achieved by Southern blot analysis. This procedure resulted in strain ATΔ*virD2* harboring a total deletion of the *virD2* gene (see Figure 3).

Construction of the omega *VirD2* mutations (Fig. 1C).

Primer prω1 (Fig. 2), containing the deletion of the omega sequence (Fig. 1B), was used in conjunction with primer pr5 to amplify the whole vector pVD43, which contained the *virD* promoter and the *virD2* gene (Rossi et al. 1993b). After sequencing the omega mutation, a *Bam*HI-*Eco*RI fragment from this mutant *virD2* gene was used to replace the corresponding fragment in plasmid pAVD43, giving rise to pA*virD2*Δω. After its introduction into the *E. coli* helper strain S17-1, pA*virD2*Δω was transferred to *Agrobacterium* strain ATΔ*virD2*, where the construct recombined with the homologous sequences in the pBR322 region of the Ti plasmid, resulting in the strain AT*virD2*Δω. The same procedure was used to construct the plasmid pA*virD2*ω/G, which contained a

glycine replacement of the omega sequence (Fig. 1B). The primer for the initial polymerase chain reaction was prω2 (Fig. 2) and after recombination the *Agrobacterium* strain AT-*virD2*ω/G was produced. The wild-type strain AT*virD2*⁺ was constructed by complementing the strain ATΔ*virD2* with the plasmid pAVD43.

T-strand assay.

The protocol has been previously described (Tinland et al. 1995). After 8 h of induction in MS medium pH 5.4 (Murashige and Skoog 1962), containing 100 mM acetosyringone, total DNA was isolated from the *Agrobacterium* strains and a non-denaturing Southern blot was performed. The T-DNA strands were detected by hybridization with a 2.2-kb *Bam*HI fragment internal to the *uidA* gene of the pTd33 plasmid (Fig. 4).

Tobacco seedling transformation assay.

A seedling assay was used to measure the transient and stable expression of genes carried by the T-DNA (Rossi et al. 1993a; Tinland et al. 1995). Four hundred seedlings were co-cultivated with *Agrobacterium* for 3 days and 30 to 90 were assayed for the transient expression of the *uidA* gene (encoding β-glucuronidase). The remaining seedlings were placed on kanamycin-containing media for the selection of stable integration of the transferred T-DNAs. The T-DNAs contained the *nptII* gene, responsible for the kanamycin resistance. The seedlings were subcultured every 2 weeks, and the resistant calli were scored after 6 weeks.

Fig. 5. Southern analysis of the right T-DNA end-plant DNA junctions with enzymes *Hind*III alone (lanes 1 to 8), and in combination with *Eco*RI (lanes 9 to 16). The probe used corresponds to an internal fragment of the *uidA* gene (Fig. 4A). Lanes 1 and 9 show the pattern obtained from a plant transformed with the wild-type strain AT*virD2*⁺; lanes 2 to 8 and 10 to 16 show the patterns obtained from seven independent transformants obtained with the omega glycine replacement mutant strain AT*virD2*ω/G.

Plant DNA extractions, Southern blot analysis.

Plant DNA was extracted as described by Paszkowski et al. (1984). Southern blot analysis was performed as described by Sambrook et al. (1989). Radioactive probes were made with a random-priming labeling kit (Boehringer-Mannheim, Mannheim, Germany) with ($\alpha^{32}\text{P}$)dATP (Amersham, Little Chalfont, England).

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LITERATURE CITED

- Bonnard, G., Tinland, B., Paulus, F., Szegedi, E., and Otten, L. 1989. Nucleotide sequence, evolutionary origin and biological role of a rearranged cytokinin gene isolated from a wide host range biotype III *Agrobacterium* strain. *Mol. Gen. Genet.* 216:428-38.
- Bundock, P., Dendulkas, A., Beijersbergen, A., and Hooykaas, P. J. J. 1995. Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *EMBO J.* 14:3206-3214.
- Gough, J., and Murray, N. 1983. Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J. Mol. Biol.* 166:1-19.
- Hooykaas, P. J. J., and Beijersbergen, A. 1994. The virulence system of *Agrobacterium tumefaciens*. *Annu. Rev. Phytopathol.* 32:157-179.
- Howard, E. A., Zupan, J. R., Citovsky, V., and Zambryski, P. C. 1992. The VirD2 protein of *A. tumefaciens* contains a C-terminal bipartite nuclear localization signal: Implication for nuclear uptake of DNA in plant cells. *Cell* 68:109-118.
- Koukolíková-Nicola, Z., Raineri, D., Stephens, K., Ramos, C., Tinland, B., Nester, E. W., and Hohn, B. 1993. Genetic analysis of the VirD2 operon of *Agrobacterium tumefaciens*: A search for function involved in the transport of T-DNA into the plant cell nucleus and in T-DNA integration. *J. Bacteriol.* 175:723-731.
- Makkerh, J. P. S., Dingwall, C., and Laskey, R. A. 1996. Comparative mutagenesis of nuclear localization signals reveals the importance of neutral and acidic amino acids. *Curr. Biol.* 6:1025-1027.
- Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Narasimhulu, S. B., Deng, X., Sarria, R., and Gelvin, S. B. 1996. Early transcription of *Agrobacterium* T-DNA genes in tobacco and maize. *Plant Cell* 8:873-886.
- Parke, D. 1990. Construction of mobilisable vectors derived from plasmids RP4, pUC18 and pUC19. *Gene* 93:135-137.
- Paszkowski, J., Shillito, R. D., Saul, M. W., Mandak, V., Hohn, T., Hohn, B., and Potrykus, I. 1984. Direct gene transfer to plants. *EMBO J.* 3:2717-2722.
- Ream, W. 1989. *Agrobacterium tumefaciens* and interkingdom genetic exchange. *Annu. Rev. Phytopathol.* 27:583-618.
- Rossi, L., Escudero, J., Hohn, B., and Tinland, B. 1993a. Efficient and sensitive assay for T-DNA dependent transient gene expression. *Plant Mol. Biol. Rep.* 12:220-229.
- Rossi, L., Hohn, B., and Tinland, B. 1993b. The VirD2 protein carries nuclear localization signals important for transfer of T-DNA to plants. *Mol. Gen. Genet.* 239:345-353.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Shurvington, C. E., Hodges, L., and Ream, W. 1992. A nuclear localization signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. *Proc. Natl. Acad. Sci. USA* 89:11837-11841.
- Simon, R., Priefer, U., and Pühler, A. 1983. A broad host range mobilisation system for *in vivo* genetic engineering: Transposon mutagenesis in gram negative bacteria. *Biotechnology* 1:784-791.
- Sonti, R. V., Chiurazzi, M., Wong, D., Davies, C. S., Harlow, G. R., Mount, D. W., and Signer, E. R. 1995. *Arabidopsis* mutants deficient in T-DNA integration. *Proc. Natl. Acad. Sci. USA* 92:11786-11790.
- Stachel, S., Timmerman, B., and Zambryski, P. 1987. Activation of *Agrobacterium tumefaciens* vir gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: Requirement for 5' virD gene products. *EMBO J.* 6:857-863.
- Stachel, S. E., and Nester, E. W. 1986. The genetic and transcriptional organization of the vir region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. *EMBO J.* 5:1445-1454.
- Steck, T. R., Lin, T., and Kado, C. I. 1990. VirD2 gene product from the nopaline plasmid pTiC58 has at least two activities required for virulence. *Nucleic Acids Res.* 18:6953-6958.
- Tinland, B. 1996. The integration of T-DNA into plant genomes. *Trends Plant Sci.* 1:178-184.
- Tinland, B., Koukolíková-Nicola, Z., Hall, M. N., and Hohn, B. 1992. The T-DNA-linked VirD2 protein contains two distinct functional nuclear localization signals. *Proc. Natl. Acad. Sci. USA* 89:7442-7446.
- Tinland, B., Schoumacher, F., Gloeckler, V., Bravo-Angel, A. M., and Hohn, B. 1995. The *Agrobacterium tumefaciens* virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. *EMBO J.* 14:3585-3595.
- Wang, K., Herrera-Estrella, A., and Van Montagu, M. 1990. Overexpression of virD1 and virD2 genes in *Agrobacterium tumefaciens* enhances T-complex formation and plant transformation. *J. Bacteriol.* 172:4432-40.
- Ward, E. R., and Barnes, W. M. 1988. VirD2 protein of *Agrobacterium tumefaciens* is very tightly linked to the 5' end of T-strand DNA. *Science* 242:927-930.
- Zambryski, P. C. 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43:465-490.