Identification of a Carboxy-Terminal Glutamine-Rich Domain in *Agrobacterium tumefaciens* Coupling Protein VirD4 Required for Recognition of T-Strand DNA and Not VirE2 as a Substrate for Transfer to Plant Cells

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Agrobacterium tumefaciens transfers DNA and proteins to a plant cell inciting crown gall tumor disease on most plants. VirD4 targets the DNA and protein substrates to a type IV secretion (T4S) apparatus for translocation into the plant cell. Several bacteria with VirD4 homologs use T4S for intercellular export of microbial macromolecules to eukaryotic and prokaryotic hosts. How the VirD4 proteins recognize the diverse substrates is not well understood. To identify functional domains of *A. tumefaciens* pTiA6 VirD4, we introduced random 19-codon and targeted 10-codon insertions throughout the coding region. Analysis of 21 mutants showed that only the carboxy-terminal end of VirD4 is tolerant of an insertion. Sequence comparison of VirD4 proteins of *Agrobacterium* spp. and their close relative, *Rhizobium etli*, showed that these proteins contain a highly conserved C-terminal end, but the immediate upstream regions share no discernible sequence similarity. The conserved region sequence is rich in the amino acid glutamine (6/13 Q). Using site-specific and deletion mutagenesis, we demonstrated that the conserved Q-rich region is required for VirD4 function and for the specific recognition of VirD2-linked T-strand DNA as a substrate for translocation to plants. The Q-rich region is not required for the transfer of a second *A. tumefaciens* substrate, VirE2, to plants or a promiscuous *Escherichia coli* IncQ plasmid to another *A. tumefaciens* strain. We identified Q-rich sequences at or near the C terminus of several VirD4 homologs, including the *E. coli* F plasmid TraD. In F TraD, the Q-rich sequence maps to a region required specifically for the conjugative transfer of the F plasmid.

Agrobacterium tumefaciens causes crown gall tumor disease on dicotyledonous plants by transferring oncogenic DNA to plant cells using a type IV secretion (T4S) apparatus (Chilton

et al. 1977; Vergunst et al. 2000). T4S is used by bacteria to transfer large macromolecules across cell membranes into other bacteria and eukaryotes (Christie 2001). Rapid evolution of multidrug-resistant bacteria can be a result of efficient horizontal transfer of plasmid DNA among bacteria using the T4S system. Human, animal, and plant pathogens use T4S to cause diseases on eukaryotic hosts. Substrate translocation during *Agrobacterium*-mediated substrate transfer requires the participation of 12 virulence proteins, VirB1 to VirB11 and VirD4 (Berger and Christie 1994; Vergunst et al. 2000). Many of these proteins are conserved in bacteria harboring a T4S system (Christie 2001; Christie et al. 2014). The VirB proteins assemble a multiprotein translocation apparatus spanning both bacterial membranes, while the coupling protein VirD4 functions by targeting a substrate to the apparatus for delivery into a recipient cell (Cabezón et al. 1997; Fronzes et al. 2009). The number of substrates translocated to a host varies widely, ranging from a few in *Agrobacterium* and *Helicobacter* species to hundreds in *Legionella* species (Christie et al. 2014; Escoll et al. 2013). Molecular mechanisms of substrate recognition by the VirD4 family of coupling proteins are not well understood.

For DNA transfer to plants, the *A. tumefaciens* Ti-plasmid is processed at the T-DNA border sequences, delineated by a conserved 24-bp direct repeat sequence at the two termini, by the VirD1 and VirD2 relaxase, producing a single-stranded T-strand DNA molecule covalently attached to VirD2 (Christie 2001). VirD4 escorts the VirD2–T-strand DNA intermediate to the T4S apparatus for secretion (Cascales et al. 2005). A few proteins, including VirD5, VirE2, VirE3, and VirF, as well as *A. rhizogenes* GALLS-FL and GALLS-CT, also are exported to a plant cell in a VirD4-dependent manner (Gelvin 2012). VirE2 is a single-stranded DNA-binding protein whose protective function against nucleases is required in the plant cell (Das 1988; Simone et al. 2001).

VirD4 is an integral membrane protein with two transmembrane (TM) domains near the N terminus (Das and Xie 1998). The small periplasmic domain is required for DNA transfer and for the localization of VirD4 to a cell pole (Kumar and Das 2002). This domain also functions in the handover of a DNA substrate from VirD4 to VirB11 and other VirB channel proteins in the transfer pathway (Cascales et al. 2013). Immediately after the second TM domain, VirD4 contains ATP-binding Walker A and B motifs that are essential for DNA transfer (Cascales et al. 2013; Kumar and Das 2002). The soluble

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domain of a VirD4 homolog, the *Escherichia coli* plasmid R388 TrwBAN70, binds ATP, which is required for the conjugative transfer of the plasmid (Tato et al. 2007). These studies suggest a role for ATP utilization in VirD4 function.

TrwBAN70 is a homo-hexameric protein with a central opening of 20 Å capable of accommodating large substrates (Gomis-Rüth et al. 2001 and 2002). A helical bundle, known as the all-alpha domain (AAD), sits at the entrance of the channel. Biochemical studies with *A. tumefaciens* VirD4 suggest that the AAD region participates in the recognition of DNA, and possibly proteins, as substrates (Whitaker et al. 2015). Members of the VirD4 protein family share significant sequence similarity throughout most of the protein, except for the extreme C terminus. The C terminus of the pTiA6 VirD4, however, is required for function (Kumar and Das 2002; Whitaker et al. 2016). This region in the VirD4 family of proteins is highly variable, both in primary sequence and in its length. These differences have led to the postulate that the region participates in recognition of system-specific substrates. A recent study using chimeric VirD4 proteins supports this hypothesis (Whitaker et al. 2016).

The introduction of short (10 to 30 amino acids) random sequences within a protein could adversely affect its function (Nelson et al. 1997). This property has been used successfully to identify protein functional domains and to introduce detection tags (Haft et al. 2007; Waters and Dunny 2001). To identify functional domains of pTiA6 VirD4, we introduced 19- and 10-codon insertions by transposon and site-specific mutagenesis, respectively. We demonstrate that only the C-terminal end of VirD4 is tolerant of an insertion. Hidden within this region we identified a conserved 13-residue glutamine-rich domain (46% Q) that is required for DNA transfer to plants. This domain is essential for the recognition of the T-strand DNA linked to VirD2 as a substrate for transfer to plants. We also show that the Q-rich domain is not required for the transfer of a second *A. tumefaciens* substrate, VirE2, to plant cells or of the promiscuous IncQ plasmid pKT230 DNA to another *A. tumefaciens* strain. Our results suggest that, at a minimum, two separate VirD4 domains are involved in the recognition of substrates targeted for translocation via a T4S system.

RESULTS

Insertional mutagenesis of *virD4*.

To probe for functional domains of *A. tumefaciens* pTiA6 VirD4, we introduced random insertions of 19 codons within the coding region of a cloned *virD_p-virD4* gene by EZ-tn5 transposon mutagenesis. Analysis of plasmid DNA from 120 kanamycin-resistant colonies by restriction mapping and DNA sequence analysis led to the identification of 11 unique insertions in the *virD4* coding region. The insertions mapped throughout the gene; however, a few regions were devoid of an insertion (Fig. 1). To fill in the gaps, 10 additional mutants with 10-codon insertions at targeted sites were generated by site-specific mutagenesis (Kumar and Das 2002). Analysis of 21 mutants covering the *virD4* coding region showed that an insertion is not tolerated in most of the protein except for the C terminus (Fig. 1). Sixteen insertions that mapped within the first 542 residues produced proteins that were nonfunctional in T-DNA transfer in complementation assays. In contrast, four insertions mapping within the C-terminal 103 residues, at codons 554, 580, 609, and 655 (the penultimate codon), had no effect on protein function. A fifth insertion mapping to this region, at codon 640 (VirD4_{i640}), did render the protein non-functional, indicating that the extreme C terminus is required for T-DNA transfer. All mutant proteins, except for two (VirD4_{i291} and VirD4_{i332}), were stably expressed in the cell (Supplementary Fig. S1).

A *virD4* mutant proficient in VirE2 transfer but deficient in T-strand DNA transfer.

T-DNA and VirE2 protein are independently transferred into a plant cell by *A. tumefaciens* (Binns et al. 1995). We studied the effect of an insertion in *virD4* on T-DNA transfer and VirE2 transfer using in-planta complementation assays (also known as extracellular complementation tests [Ottens et al. 1984]). All avirulent mutants, except for one, were deficient in the independent transfer of the two substrates (Fig. 1). The exception was VirD4_{i640}, a mutant deficient in T-DNA transfer but proficient in VirE2 transfer (Fig. 1). The novel phenotype of VirD4_{i640} suggests that the C terminus of VirD4 encodes a VirD2-linked T-strand DNA recognition motif and that this motif is not

Fig. 1. Insertional mutagenesis of *virD4*. An insertion at *virD4* codon “n” is indicated by a vertical line. The two transmembrane domains (dark boxes) and the Walker A motif (*) are also shown. Lines above the box identify insertions obtained by EZ-tn5 mutagenesis, and lines below identify those introduced by site-specific mutagenesis. The effect of a mutation on VirD4 function was monitored by complementation of the *virD4* deletion mutant *Agrobacterium tumefaciens* A348ΔD4. The effect on either T-strand DNA or VirE2 transfer to plants was monitored by in planta complementation assays by coinoculation with *A. tumefaciens* MX358 (*virE2*⁺) or PC2760 (T-DNA⁺). -: avirulent; +: virulent. Insertions at codons 554, 580, 609, and 655 are proficient in both T-DNA and VirE2 transfer based on their virulent phenotype in *virD4* complementation assays.

required for the transfer of the second substrate, VirE2. The recognition motifs for the two substrates, therefore, are likely to be different.

A conserved glutamine-rich region at the C terminus of *Agrobacterium* sp. VirD4 proteins.

The unique phenotype of VirD4_{i640} prompted us to take a closer look at the primary sequences of the C-terminal end of this family of proteins. All VirD4 family members share significant sequence similarity throughout the protein except in the C-terminal region, where similarity is virtually non-existent (Supplementary Fig. S2). Diversity in the C-terminal sequences could provide the flexibility necessary for the recognition of system-specific substrates exported by the VirD4 homologs. This region, thus, has been postulated to function in substrate recognition, and a recent study supports this hypothesis (Whitaker et al. 2016). Substrates transferred by the pathogenic *Agrobacterium* sp., however, are similar, and therefore, the conservation of a substrate recognition motif is expected in this subfamily. To test this hypothesis, we compared the primary sequences of the C-terminal 100 residues (the only region with several silent insertions [Fig. 1]) of VirD4 proteins of *Agrobacterium* sp. and a close relative, *Rhizobium etli*. Surprisingly, the proximal (N-terminal) approximately 70 residues showed no discernible sequence similarity, but the C-terminal approximately 30-amino acid region was highly conserved (Fig. 2; Supplementary Fig. S3). These findings, in conjunction with the phenotype of the mutants discussed previously, led us to postulate that a substrate recognition site resides at the extreme C terminus of VirD4 proteins and that the immediately upstream region serves as a spacer.

A unique feature of the C-terminal conserved region (residues 624 to 646 in pTiA6 VirD4) is that it contains a small segment rich in the amino acid glutamine (Fig. 2). Within a 13-amino acid region, residues 633 to 645, six are glutamine residues (hereafter referred to as the Q-rich domain). Interspersed among the glutamine residues are five invariant leucine and isoleucine residues. Two invariant alanine and arginine residues border the N- and C-terminal sides, respectively, and several additional conserved residues are present on the N-terminal side of the Q-rich domain.

Functional analysis of the Q-rich domain.

Mutational studies were performed to determine the role of the Q-rich domain in T-DNA transfer. We introduced amino acid substitutions and small deletions within the Q-rich domain and studied the effect of a mutation on VirD4 function by complementation assays. Alanine substitutions of the invariant Q633 (Q633A) and of both invariant Q644 and near invariant Q645 (QQ64445AA) abolished VirD4 function (Fig. 3A and B). A triple mutant with glutamine to asparagine substitution of residues 633 to 635 (QQQ633-35NNN) exhibited near avirulent phenotype. This mutant formed a few small tumors only after prolonged infection (10 to 12 weeks postinfection). Two double mutants with alanine substitutions of the two invariant leucine residues at position 636 and 637 (LL63637AA) and the two invariant isoleucine residues at 640 and 641 (II64041AA) also were nonfunctional. A substitution of aspartic acid 638 to valine (D638V), on the other hand, had no effect on VirD4 function. The conserved residues within the Q-rich domain, therefore, are required for VirD4 function.

To map the domain more precisely, we analyzed five deletion mutants (Fig. 3A and B). Deletion of six codons (633 to 638) within the first half of the Q-rich domain rendered the protein nonfunctional. Deletion of three amino acids at the other end, codons 646 to 648, had no effect on VirD4 function. Two deletions in the extreme C terminus were introduced by deleting codons 631 to 656 and by changing codon 648 to a stop codon (resulting in the deletion of codons 648 to 656; R648X). In complementation assays, VirD4Δ631-656 had no DNA transfer activity, while VirD4R648X was fully functional. The last nine nonconserved residues of VirD4 are not required, therefore, for protein function and are not part of the Q-rich domain.

Sequences upstream of the Q-rich sequences are not conserved among the Ti- and Ri-plasmid VirD4s (Supplementary Fig. S3). To investigate the role of these sequences in VirD4 function, we introduced a large deletion of codons 561 to 620. The mutant protein, VirD4Δ561-620, was fully functional in tumor formation (Fig. 3B). T-DNA transfer thus requires VirD4 residues 631 to 646, which include a conserved Q-rich domain, and its immediately upstream region has no role in VirD4 function.

pTiA6	621	VVPPELTLALAAQQQLLDQI IALQQRSRSAPAQPAK	656
pTiBo542	621	VVPPELTLALTAQQQLLDQI IALQQRSRSAPA----	652
pTiC58	630	GVSAEMAPAMIAQQQLLEQI IALQQRYGPASSHSVK	665
pTi-Sakura	633	GVSAEMAPAMIAQQQLLEQI IALQQRYGPASSHSVK	668
<i>A. vitis</i>	631	EELLDIAPALLAQREELLEQI ISLQQRGGTDSGT---	663
pRiA4b	636	SVPAEMAPALSAQQQLLGQI IALQQRYRPVSSNP IE	671
pRi1724	628	EKLPEIAPALLAQRELLDQI ISLQLRGRTASGT---	660
<i>R. etli</i>	631	GPSAEVAPALVAQEQLLKRI IALQQRYRSGSSNP GD	666
		: :: * : ** . : ** : ** * .	
consensus		pebapAl AQqqLL+qIIaLQqR <u>r</u> s	
		a g	

Fig. 2. Sequence conservation near the C terminus of *Agrobacterium* and *Rhizobium* spp. VirD4 proteins. Sequence similarity near the C terminus of the closely related VirD4 proteins is shown. A consensus sequence with conserved residues in capital letters and near conserved residues (≥6/8) in small letters is also shown. *: invariant; +: acidic; b: bulky. No sequence conservation was observed within the preceding 70 amino acids. pTiA6 (accession number NP_059816.1), pTiBo542 (AAZ50535), pTiC58 (AAA91606.1), pTi-Sakura (NP_053398.1), *A. vitis* S4 (ACM39656.1), pRiA4b (P13464.1), pRi1724 (NP_066751.1), and *R. etli* CFN 42 (YP_471662.1).

We used in planta complementation assays to investigate whether the inability of a mutant protein to complement the *A. tumefaciens* deletion strain A348DD4 was due to a defect in the transfer of T-strand DNA, VirE2, or both substrates. All complementation-deficient mutants failed to transfer T-strand DNA but were fully competent in VirE2 transfer (Fig. 3A and C). The VirE2 transfer-proficient phenotype of these mutants and that of VirD4Δ631-656, a mutant with a complete deletion in the Q-rich domain, indicates that different VirD4 domains participate in recognition of the two substrates. The Q-rich domain is required for the recognition of the VirD2 linked T-strand DNA substrate but not VirE2. The VirE2 recognition domain, therefore, must lie elsewhere in the protein.

Effect of mutations in *virD4* on conjugative transfer of IncQ plasmid to another *A. tumefaciens* strain.

The *A. tumefaciens* T4S system supports conjugative transfer of promiscuous IncQ plasmids into another bacterium (Beijersbergen et al. 1992). We studied the effect of the mutations in *virD4* on conjugative transfer of plasmid pKT230 to another *A. tumefaciens* strain. All insertion mutants that were defective in T-DNA transfer to plants, except for *virD4*_{i640}, were also defective in pKT230 transfer to the recipient bacterium (Fig. 4). *virD4*_{i640}, which is deficient in T-DNA transfer, was competent in pKT230 DNA transfer. All mutants mapping to the Q-rich domain also were proficient in plasmid DNA transfer. One mutant, *virD4*Q633A, was less efficient in pKT230 DNA transfer. The Q-rich domain and the C-terminal end of VirD4, therefore, are not required for the transfer of IncQ plasmid DNA to another bacterium, and a second DNA

recognition domain must be present within the first 554 residues of the protein.

DISCUSSION

The VirD4 coupling proteins target DNA and protein substrates to the T4S apparatus for transfer to a recipient cell.

Fig. 4. Effect of a mutation in *virD4* in conjugative transfer of IncQ plasmid pKT230 is shown. Transfer of plasmid pKT230 from a *virD4* mutant strain to another *A. tumefaciens* strain was monitored. VirD4_{i640} and all Q-rich domain mutants were proficient in DNA transfer. VirD4Q633A exhibited a low efficiency of transfer. Data presented are an average of two independent experiments.

Fig. 3. Role of the VirD4 C-terminal Q-rich region in substrate recognition. **A**, Effect of amino acid substitutions and deletions within the Q-rich domain on VirD4 function was analyzed by complementation assays. *: Invariant residues; -: deletion; x: stop codon; +: proficient; -: no transfer; ±: very weakly proficient. **B**, Phenotype of mutants in *virD4* complementation assays is shown. +: A348; -: A136. **C**, Ability of a *virD4* mutant to transfer T-DNA (+LBA4404 [L]) or VirE2 (+MX358 [M]) by in planta complementation assays is shown. AD1326 (*virD4*Q633A), 1327 (LL63637AA), 1329 (II64041AA), 1324 (QQ64445AA), 1323 (Δ633-638), 1334 (Δ631-656).

Mechanisms of substrate recognition and secretion are largely unknown. Protein–protein interaction is expected to play a primary role in substrate recognition. Functional analysis of *A. tumefaciens virD4* by insertional mutagenesis showed that more than three-fourths of the N-terminal region of the protein could not be modified without a loss of the DNA transfer function (Fig. 1). Several mutants we analyzed had an insertion of 10 amino acids, a short insertion commonly tolerated by proteins. The insertions mapped primarily to four regions: the periplasmic domain (approximately aa 31 to 67) (VirD4_{i62}); the nucleotide-binding Walker B motif (VirD4_{i395}); the AAD domain (approximately aa 201 to 346, according to [Whitaker et al. 2015]) (VirD4_{i200}, VirD4_{i237}, VirD4_{i260}, VirD4_{i272}, and VirD4_{i316}); and the inner channel (VirD4_{i430} and VirD4_{i464}), membrane-proximal channel end (Gomis-Rüth et al. 2001) (VirD4_{i93} and VirD4_{i490}) and C-terminal end (VirD4_{i526}, VirD4_{i542}, VirD4_{i554}, VirD4_{i580}, VirD4_{i609}, VirD4_{i640}, and VirD4_{i655}). Only four insertions (VirD4_{i554}, VirD4_{i580}, VirD4_{i609}, and VirD4_{i655}), all mapping to the C-terminal end of the protein, did not abolish protein function.

Three mutations, two avirulent (VirD4_{i526} and VirD4_{i542}) and one virulent (VirD4_{i554}), define the end of the conserved region among the VirD4 homologs. The avirulent mutations mapped to a highly charged region. Within a 34-residue segment (aa 509 to 542), 14 are charged residues (nine basic, five acidic). The equivalent region in TrwB is not charged but is required for function, because a deletion of the C-terminal 12 or 17 residues led to severe reduction (3 to 4 log) in its DNA transfer activity (de Paz et al. 2010). Future studies will determine whether the polarity of the region or the secondary structure plays a role in VirD4 function.

The extreme C terminus of the VirD4 proteins is highly variable and is postulated to encode system-specific function. We demonstrate that this region of *Agrobacterium* sp. and *R. etli* VirD4 proteins contains a conserved Q-rich sequence that is essential for recognition of the VirD2-T-strand DNA as a substrate for translocation to plants (Figs. 2 and 3). This domain is not required for the recognition of a second *A. tumefaciens* substrate, VirE2, and that of promiscuous IncQ plasmid DNA substrate. Thus, the Q-rich domain is a specificity factor solely for the recognition of VirD2-T-strand DNA substrate. The fact that the VirD4 Q-rich domain mutants with amino acid

substitutions or deletions were fully competent in VirE2 transfer suggests that a second substrate recognition site lies elsewhere in the protein (Fig. 3). The AAD domain has been proposed to function in substrate recognition (Whitaker et al. 2015).

We envision that the AAD serves as the primary substrate recognition domain. This is consistent with observations in this study and by Whitaker et al. (2016) that several VirD4 homologs (e.g., R388 TrwB, pKM101 TraJ, and *Sinorhizobium medicae* VirD4 [WP_011970866.1]) lack an additional C-terminal domain; that in vitro a protein containing only the AAD of *A. tumefaciens* VirD4 specifically bound VirD2; and that VirD4 mutants lacking the only essential domain in the C-terminal region, the Q-rich domain, are proficient in the transfer of both VirE2 and IncQ plasmid DNA. We propose that the Q-rich domain modulates the primary substrate recognition domain, enabling it to recognize other substrates. The Q-rich domain probably interacts directly with the VirD2 relaxase to target VirD2-T-strand DNA to VirD4 or to stabilize interaction between VirD4 AAD and VirD2 linked to the T-strand DNA.

A low efficiency of VirD4Q633A in IncQ DNA transfer is consistent with the hypothesis that the Q-rich domain participates in protein–protein interaction. This domain is not required for IncQ plasmid DNA transfer, since VirD4Δ631–656, a mutant with a complete deletion of the domain, as well as all mutants mapping to the Q-rich domain, except for VirD4Q633A, were proficient in pKT230 DNA transfer. The simplest explanation for the VirD4Q633A phenotype is that interaction of the mutated Q-rich domain with the DNA transfer domain is inhibitory to pKT230 transfer. The dispensable nature of the VirD4 Q-rich domain for the recognition of VirD2 in vitro reported previously (Whitaker et al. 2016) could be due to expression of the AAD in isolation. In the absence of the other domains, VirD4 AAD could acquire a conformation proficient in interaction with VirD2.

In addition, two VirD4 homologs, TraD of the *E. coli* R and F plasmids, contain C-terminal protein–protein interaction domains necessary for conjugative transfer of plasmid DNA (Beranek et al. 2004; Lu and Frost 2005). The C-terminal 38 residues of R plasmid TraD interact with the TraM relaxosome protein. In F-TraD, the C-terminal 37 amino acids (residues 680 to 717) offer specificity for F-plasmid transfer, most likely

Protein (Size, aa)	Source	Accession #	Q-rich Region Sequence	% Q
TraD (716)	F plasmid – <i>E. coli</i>	AAA83928	681 QQENHPDIQQQMQ 693	46
VirD4 (639)	<i>B. henselae</i>	AFK10358	593 QEMRQDEQIILMQ 605	30
VirD4 (754)	<i>H. pylori</i>	EJB25423	738 QQGDEESEQEVLQDTQ 753	31
VirD4 (649)	<i>Roseibium</i> sp.	EFO28786.1	628 QAKADLQLEKALQVRQ 643	25
TraD (705)	<i>V. vulnificus</i>	BAC97747.1	680 QERAQQELDEAAISQDIQ 697	28
VirD4 (382)	<i>A. faecalis</i>	EJC62462.1	352 QFQPFAQVIEPFIESNQ 368	24
TraD (556)	<i>C.</i> <i>amoebophila</i>	CAF24165	515 QQTRYQQKITKTDIQFLSRNQ 535	29

Fig. 5. Q-rich regions near the C terminus of VirD4 homologs are shown. Glutamine residues are shown in bold.

through an interaction with the cognate relaxosome (Sastre et al. 1998). The extreme C terminus of F-TraD (residues 703 to 717) is probably not required for F-transfer, because a 31-amino acid insertion at residue 702 of F-TraD was transfer proficient (Nelson et al. 1997). The essential C-terminal F-TraD domain, therefore, is likely to lie within residues 680 to 702 (WQQENHPDIQQMQRREEVNINV) (Fig. 5). This sequence, like that in VirD4, is rich in glutamine (6 Q/13). Several other VirD4 homologs also contain a Q-rich domain (discussed below).

Q-rich domains also are known to participate in interactions among eukaryotic proteins. Eukaryotic transcription activators, like human transcription factor Sp1, contain Q-rich domains with a hydrophobic patch (Gill et al. 1994). This domain in human Sp1 is involved in protein–protein interactions required for the assembly of the transcription factor TFIID (Gill et al. 1994; Rojo-Niersbach et al. 1999; Tanese et al. 1996). We propose a similar role for the VirD4 Q-rich domain in interaction with the VirD2 relaxase or a T4S substrate. The essential nature of multiple glutamine and bulky hydrophobic residues in VirD4 function support this hypothesis (Fig. 3). The Q-rich domain identified in this study is not unique to the *Agrobacterium* sp. VirD4 proteins and F TraD. Manual analysis of the C-terminal end sequences of VirD4 homologs showed that a Q-rich domain is present at or near the C-terminus of several proteins, including *Bartonella henselae* VirD4, *Helicobacter pylori* VirD4, *Rosebium* sp. VirD4, the marine pathogenic γ -proteobacterium *Vibrio vulnificus* TraD, β -proteobacterium *Alcaligenes faecalis* VirD4, and Chlamydiae *Protochlamydia amoebophila* TraD (Fig. 5). The *B. henselae* Q-rich domain, like that of VirD4_{At}, contains large hydrophobic spacer residues. In some homologs (e.g., *Wolbachia pipientis* VirD4), the C-terminal end is very acidic and is rich in aspartic acid (Whitaker et al. 2016). The role of these domains in substrate recognition remains to be determined.

MATERIALS AND METHODS

Strains and plasmids.

A. tumefaciens A136 is a derivative of strain C58 cured of its Ti-plasmid pTiC58 (Chilton et al. 1977). *Agrobacterium* sp. strain A348, a derivative of *A. tumefaciens* A136, harbors the octopine Ti-plasmid pTiA6NC. Strain MX358 is an *A. tumefaciens* A348 derivative with a transposon insertion in virE2, and *A. tumefaciens* LBA4404 has a deletion in the T-DNA region of the Ti-plasmid pTiAch5 (Hoekema et al. 1983; Stachel and Nester 1986). *A. tumefaciens* A348/pKT230 and A348/pJB61 harbor IncQ plasmid pKT230 (kanamycin resistant) and plasmid pJB61 (gentamycin resistant), respectively (Beijersbergen et al. 1992; Bohne et al. 1998).

A. tumefaciens was grown overnight at 30°C in AB minimal medium with the appropriate antibiotic. For induction of virulence genes, bacteria were grown at 22°C in AB Mes pH 5.8 minimal medium supplemented with 100 μ M acetosyringone as described previously (Kumar and Das 2002).

Construction of *A. tumefaciens* virD4 deletion mutant strain A348 Δ D4.

An unmarked deletion in the pTiA6NC *virD4* was introduced by homologous recombination using *sacB* selection (Skerker and Shapiro 2000). DNA fragments of 1.1 kb (*Bam*HI-*Bsp*HI fragment) and 1.5 kb (*Eco*RV-*Bam*HI fragment) from the left and right ends of *virD4*, respectively, were cloned into the *sacB* vector plasmid pNPTS138. The resultant plasmid, pPU20, was introduced into *A. tumefaciens* A348 by electroporation (Mersereau et al. 1990), and sucrose-resistant colonies were isolated. Several sucrose-resistant colonies were analyzed by

Southern blot hybridization and PCR amplification. A colony with the desired deletion in *virD4* (codons 1 to 596 deleted) was named *A. tumefaciens* A348 Δ D4 and was used in all studies.

virD4 mutagenesis.

Random in-frame 19-codon insertions within the *virD4* coding region were introduced by Ez-tn5 mutagenesis (Epicentre Technologies, Madison, WI, U.S.A.). Plasmid pAD1791, a pUC119 derivative with a chimeric *virDp-virD4* gene, was mutagenized in vitro with Ez-tn5 < *Not*I/KAN-3 > transposon, as per the manufacturer's instructions. Plasmids with insertions in *virD4* were identified by restriction endonuclease digestion. The site of insertion was determined by DNA sequence analysis. Eighteen unique insertions were identified from analyses of 62 mutants. The inserted transposon was then deleted by digestion of plasmid DNA with the restriction endonuclease *Not*I, and the plasmid was recircularized with T4 DNA ligase. This process introduced an in-frame insertion of 19 codons at the original site of the transposon insertion.

Targeted insertions of 10 in-frame codons containing a flag epitope (IDYKDDDDKL) and mutations within the Q-rich domain were introduced by deoxy-oligonucleotide directed site-specific mutagenesis, using single-stranded pAD1791 DNA as a template (Kumar and Das 2002). The DNA sequence of oligonucleotides used for the introduction of mutations is listed in Supplementary Table S1. All plasmids were fused to the wide host range plasmid pAD1085 for analysis in *A. tumefaciens*. Plasmid pAD1085 was constructed by cloning a 0.7-kb *Dde*I fragment containing the pGEM1 polylinker region into the *Hind*III site of the wide host range pRK2 derivative plasmid pTJS75 (Schmidhauser and Helinski 1985). Plasmids were introduced into *A. tumefaciens* by electroporation.

virD4 functional assays.

The effect of a mutation in *virD4* on DNA transfer was monitored by complementation assays. The ability of a mutant to complement the *virD4* deletion mutant *A. tumefaciens* A348 Δ D4 was monitored by tumor-formation assays on *Kalanchoe daigramontiana* leaves (Kumar and Das 2002). Tumors were scored 3-weeks postinfection.

The effect of a mutation on T-strand DNA transfer or VirE2 transfer was monitored by in-planta complementation (Otten et al. 1984). In this assay, a wound site is coinfecting with two *A. tumefaciens*—one serving as the T-DNA donor and the other serving as the VirE2 donor. In one experimental setup, the *virD4* mutant served as the T-DNA donor, and *A. tumefaciens* LBA4404 (*vir*⁺T⁺ [Hoekema et al. 1983]) was the VirE2 donor. In the complementary set, the *virD4* mutant served as the VirE2 donor, and *A. tumefaciens* MX358 (*virE2*⁺T⁺ [Stachel and Nester 1986]) was the T-DNA donor. Equal numbers of the two bacterial strains grown overnight in AB liquid medium were mixed in a microfuge tube and centrifuged to pellet the cells. Cells were resuspended at a concentration of 1 OD/ml. Five microliters of the cell mixture was used to infect a leaf wound site.

Conjugative transfer of an IncQ plasmid between *Agrobacterium* sp. strains.

VirD4/VirB-dependent transfer of plasmid pKT230 from donor *A. tumefaciens* DD4/pvirD4 (or its mutant) into recipient *A. tumefaciens* A348 (gentamycin resistant) was monitored, essentially as described by Bohne et al. (1998). pKT230 is a kanamycin-resistant derivative of the wide host range IncQ plasmid RSF1010 (Beijersbergen et al. 1992). Donor and recipient cells were mixed (at a 1:5 ratio) and incubated for 3 days at room temperature (22°C) on plates with AB induction medium containing 100 μ M acetosyringone. The cell mixture was plated on LB plates containing kanamycin/kanamycin and

carbenicillin (to monitor donor cell concentration), gentamycin (recipient), or kanamycin and gentamycin (transconjugants). Transfer frequency is defined as the number of transconjugants recovered per donor bacterium.

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