The Plant Journal (2000) **23**(1), 11–28

REVIEW

The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights

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This review is dedicated to Jeff Schell, one of the founders of modern 'Agrobiology', the genetic and molecular dissection of crown gall disease. Together with notable scientists at the University of Gent, Belgium, Jeff spearheaded the discovery of the Ti-plasmid. The elusive 'tumor inducing principle' was uncloaked and provided impetus for an incredibly fruitful subsequent 25 years of analyses. Scientists all over the world were caught up in unraveling the underlying mechanisms of *Agrobacterium*-mediated gene transfer to plants, and along the way uncovered a movable feast of fundamental insights. Below we summarize a sampling of *Agrobacterium's* most recently recognized accomplishments.

Introduction

The soil phytopathogen *Agrobacterium tumefaciens* has been extensively studied since 1907, when as *Bacterium tumefaciens*, it was identified as the causative agent of crown gall disease (Smith and Townsend, 1907). This disease is characterized by the tumorous growth of plant tissues in the stem, and is a significant problem in the cultivation of grape vines, stone fruit and nut trees. The first indication of the cellular or biochemical mechanisms involved in tumorigenesis coincided with the discovery of the plant growth regulator auxin. *Agrobacterium*-induced tumors were shown to be sources of auxin (Link and Eggers, 1941), and capable of growth in culture in the absence of both bacteria as well as the complement of plant growth regulators normally required to incite growth of callus from sterile plant tissues (White and Braun, 1941). Cytokinin was identified as a plant growth regulator in 1955 and shortly thereafter was strongly implicated in the growth of *Agrobacterium*-induced tumors (Braun, 1958). Braun first proposed that *Agrobacterium* was the source of a 'tumor inducing principle', possibly DNA, that permanently transformed plant cells from a state of quiescence to active cell division (Braun, 1947; Braun and Mandle, 1948). The transforming principle, however, remained elusive. With the advent of molecular techniques came the first evidence that crown gall tumors, cultured axenically, contained DNA of bacterial origin (Schilperoort *et al.*, 1967), although this conclusion was debated. Identification of the tumor-inducing (Ti) plasmid (Van Larebeke *et al.*, 1974; Van Larebeke *et al.*, 1975; Zaenen *et al.*, 1974) narrowed the search to genetic elements derived from this plasmid and ultimately resulted in the discovery of T-DNA (transferred DNA), a specific segment transferred to plant cells (Chilton *et al.*, 1977; Chilton *et al.*, 1978; Depicker *et al.*, 1978). Braun (1982) provides an interesting historical review of early work on *Agrobacterium tumefaciens*, notably studies conducted prior to the advent of molecular techniques.

Presumably, elicitation of tumors provides *Agrobacterium tumefaciens* with some advantage. This advantage derives from the production by tumors of unusual amino acid-like compounds called opines (reviewed in Dessaux *et al.*, 1993). Although opines are structurally diverse, a tumor produces only certain opines that are strictly dependent on the infecting strain, and the opines produced by a gall are specifically catabolized by that strain of *Agrobacterium* (Goldman *et al.*, 1968; Petit *et al.*, 1970). Furthermore, the ability to metabolize opines is tightly correlated with virulence; loss of virulence is always accompanied by the loss of the ability to degrade a specific opine (Petit and Tourneur, 1972). Before the identification of the Ti-plasmid, these observations were the first indication that tumorigenesis involved the transfer of genetic material from bacteria to plants. It is now known that the enzymes for catabolism of specific opines are encoded on the Ti-plasmid and complement the opine biosynthetic pathways encoded on the T-DNA. Thus, by the introduction of genetic material into plant cells, 'genetic colonization' (Schell *et al.*, 1979), *Agrobacterium tumefaciens* creates a unique habitat wherein it solely is genetically equipped to utilize the predominant carbon-nitrogen source.

Overview of *Agrobacterium*-mediated gene transfer

The tumorous transformation of plants by *Agrobacterium* results from the stimulation of plant cell division by gene products encoded by a segment of DNA (T-DNA) trans-

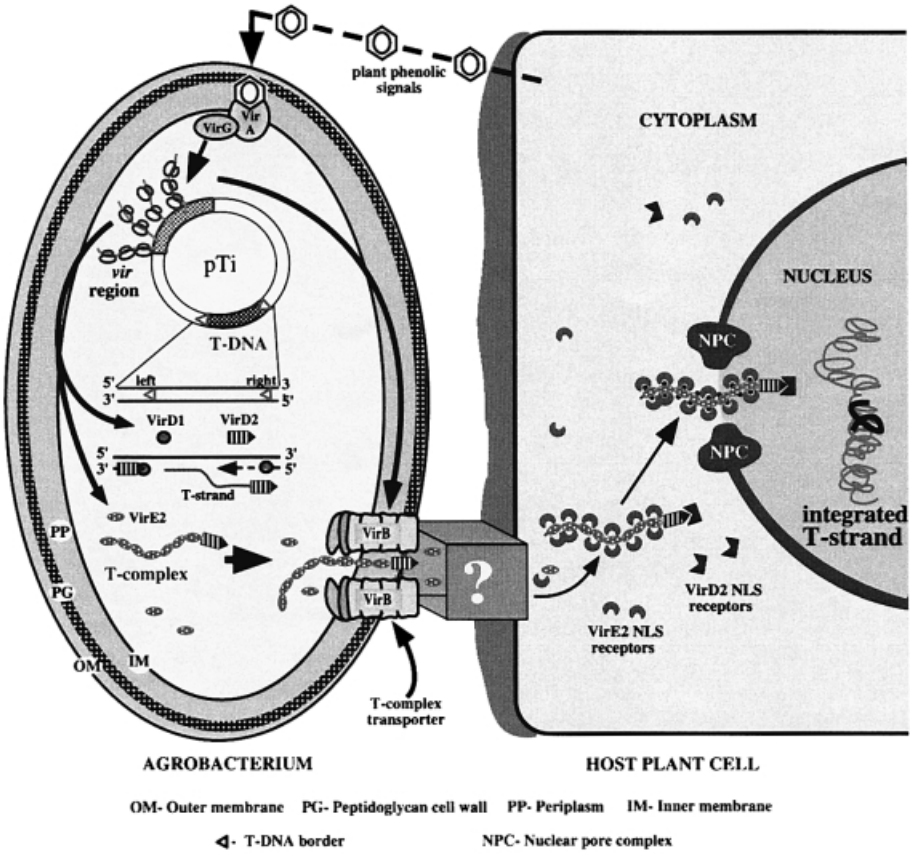

Figure 1. Basic steps in the transformation of plant cells by *Agrobacterium tumefaciens* (see text for details). Adapted from Sheng and Citovsky (1996).

ferred from the bacterium to the plant (Figure 1). The T-DNA and the virulence (*vir*) region, whose products generate the transfer intermediate (T-complex) and mediate its transport, are located on the tumor-inducing plasmid (pTi). The expression of genes in the *vir* region is induced by the exudate from wounded plants. Phenolics, such as acetosyringone, are the most potent inducers found in wound exudate, but sugars and acidic pH amplify the response. The T-complex comprises a single strand (ss) copy of the T-DNA (T-strand) with a single molecule of the Vir protein VirD2 covalently bound to the 5′ end, and coated along its length with the ssDNA binding protein, VirE2. The *vir* system of *Agrobacterium* will process and transfer any DNA between the flanking 25 bp direct repeats (right and left borders) that delimit the T-DNA; hence, the utility of *Agrobacterium* for the genetic engineering of plants. A *vir*-specific apparatus, the T-complex transporter, mediates transfer of the T-complex from the bacterium to the plant cell and is assembled from 12 membrane-associated, *vir*-specific proteins. Inside the plant cell, the T-complex is imported into the nucleus where the T-strand becomes stably integrated into a plant chromosome (reviewed in Christie, 1997; Sheng and Citovsky, 1996; Zambryski, 1997).

Fundamental insights into biological processes

Throughout its study, *Agrobacterium tumefaciens* has both spurred and benefited from advances in numerous biological processes. Table 1 highlights 12 fundamental insights derived from the analysis of the interaction between *Agrobacterium* and susceptible plant cells; selected references are listed in Table 1. To provide focus to the present review, we discuss T-DNA transfer in particular (Table 1, items 7–12). The first section focuses on analysis of the T-DNA element and attempts to place its processing and transport in a broader context. The second half of the review highlights current research on *Agrobacterium* (Table 1, item 12) and again illustrates its continued utility as a model experimental system.

Interkingdom 'conjugal' DNA transfer (Table 1, item 7)

Discovery of the T-strand directly provoked the hypothesis that the transfer of DNA from *Agrobacterium* to the plant cell is not mechanistically unique, but might be evolutionarily related to bacterial conjugation (Stachel and Zambryski, 1986a; Stachel et al., 1986b). Comparison of the synthesis of conjugal DNA transfer intermediates and

Table 1. Insights into fundamental biological processes derived from research on *Agrobacterium tumefaciens*

Insights*
1. **Communication** between microbes and plants in the soil environment: Identification of plant phenolics as *vir* inducers (Stachel *et al.*, 1985; Stachel *et al.*, 1986a).
2. VirA/VirG bacterial **two-component** system for transcriptional regulation (Stachel and Zambryski, 1986b; Winans *et al.*, 1986).
3. **Plant promoters:** Characterization of nopaline and octopine synthase promoters as two of the first plant promoters (De Greve *et al.*, 1982; Depicker *et al.*, 1982).
4. Novel pathways for **plant hormone** synthesis: T-DNA encoded genes for auxin and cytokinin biosynthesis responsible for tumor phenotype (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984; Buchmann *et al.*, 1985; Inze *et al.*, 1984; Schröder *et al.*, 1984; Thomashow *et al.*, 1984).
5. **Quorum sensing** for transcriptional regulation of conjugation: Opines stimulate conjugation of Ti-plasmid when cell density rises above some threshold (Fuqua and Winans, 1994; Fuqua *et al.*, 1994; Piper *et al.*, 1993; Zhang *et al.*, 1993).
6. Biotechnology: **Vectors** for plant transformation (Bevan, 1984; Hoekema *et al.*, 1983; Zambryski *et al.*, 1983).
7. **Interkingdom 'conjugal' DNA transfer**: Identification of T-strand transfer intermediate.
8. Evolutionary conservation of ***nic* sites** (T-DNA borders as sites of initiation and termination for T-strand production) and **nicking enzymes** (VirD1 and VirD2 together produce ss nicks in T-DNA borders).
9. Trafficking of nucleic acids: VirE2 is a **single strand nucleic acid binding protein (SSB)**.
10. **Plant nuclear localization signals:** VirD2 (and subsequently VirE2) reveal sequence requirements for plant cell nuclear import.
11. Non-**homologous recombination**: Analysis of T-DNA insertion into plant DNA.
12. **Type IV secretion systems**: The VirB T-complex transporter paradigm.

*Selected primary references for insights 1–6 (as these are not discussed in the text); see text for references related to insights 7–12.

the T-strand revealed extensive similarities. First, short nucleotide sequences required in *cis* are functionally polar in directing DNA transfer. Second, transfer is initiated at ss nicks in these motifs by sequence and strand-specific relaxases. Single-stranded, linear DNA is transferred from donor to recipient following its displacement from the plasmid. This evolutionary link was further supported by the discovery that transfer from donor to recipient of T-complex and conjugal DNA of several incompatibility (Inc) groups is mediated by an apparatus assembled from very similar proteins, encoded by operons conserved among several conjugation as well as protein export systems, designated type IV secretion systems (Christie, 1997; Lessl and Lanka, 1994; Winans et al., 1996).

Prior to defining the ss nicking reaction that initiates T-DNA transfer (below), whether the T-DNA is transferred in an ss or ds form was much debated (reviewed in Zambryski, 1992). The argument was put to rest by two very different strategies that assayed the nature of the T-DNA copy upon arrival in the plant cell. Yusibov *et al.* (1994) detected a PCR-amplified, T-DNA homologous segment within hours after *Agrobacterium* infection of tobacco protoplasts; if the plant cytoplasmic fraction first was first treated with an ss-specific nuclease, the T-DNA signal was lost. Secondly, a sensitive extrachromosomal recombination assay was employed (Tinland *et al.*, 1994), in which *in planta* recombination of the T-DNA was required to yield a full length copy of the reporter gene *uidA* (β-glucuronidase) from two overlapping coding fragments separated by an insertion. While recombination would produce an intact gene regardless of fragment polarity if the transfer intermediate is ds, a complete *uidA* can be obtained through recombination only from segments of opposite polarity if the transfer intermediate is ss. β-glucuronidase activity in infected protoplasts was an order of magnitude greater from the T-DNA bearing *uidA* segments of opposite polarity relative to segments of the same polarity. Thus, both studies provide strong confirmation for an ss transfer intermediate.

Nic *sites and nicking enzymes (Table 1, item 8)*

In type IV secretion systems that transfer DNA, synthesis of the transfer intermediate is initiated by strand-specific nicks in particular sequences, *oriT* for conjugation and T-DNA right border for T-strand (Stachel *et al.*, 1986b; Wang *et al.*, 1987). Four groups of transfer origins can be distinguished by sequence analysis (reviewed in Pansegrau and Lanka, 1996). The largest group is IncP, which includes the origins of transfer of all IncP plasmids, T-DNA borders, transfer origins from conjugative transposons, vegetative replication origins of plasmids from Gram-positive bacteria, and replication origins from ss bacteriophages. The origin for conjugal transfer of the entire Ti-plasmid between agrobacteria belongs to the IncQ group. Differences among groups in conserved nucleotides at the *nic* site indicate that the DNA:protein interactions required for substrate recognition may vary. The nicking reactions, however, probably proceed by a similar molecular mechanism, as all form a covalent bond between the cleaving enzyme and the nucleotide on the 5′ side of the *nic*. Thus, processing of nucleic acid inter-

mediates for transfer to a recipient probably originated with the evolution of specific sequence substrates for ss nicking.

During nicking, a relaxase breaks the phosphate bond between a specific pair of nucleotides in one strand of the *nic* site. In most systems, specificity for binding of the relaxase to the *nic* site is provided by an auxiliary protein which recruits the relaxase to the *nic* site (reviewed in Pansegrau and Lanka, 1996). In others, however, *nic* site recognition and cleavage functions are combined in a single protein (Pansegrau and Lanka, 1996). In *Agrobacterium*, VirD1 likely first recognizes and binds the T-DNA border to promote binding of VirD2 relaxase (Lessl and Lanka, 1994; Pansegrau and Lanka, 1996). The complex of supercoiled plasmid and nicking proteins for bacterial–bacterial conjugation systems, termed the relaxosome, is present throughout the cell cycle. In the absence of contact with a suitable recipient, the reaction equilibrium of the relaxosome does not favor the cleavage reaction. Once a recipient is physically contacted, a signal is transmitted to the relaxosome that alters the equilibrium to initiate cleavage, followed by synthesis of a transfer intermediate. *Agrobacterium* employs transcriptional regulation of VirD1 and VirD2 to ensure that a pTi-relaxosome (Filichkin and Gelvin, 1993) is assembled and active only in the presence of a plant susceptible to infection. Generally, homologies among relaxases parallel homologies of the transfer origins.

In vivo in *Agrobacterium*, VirD1/D2 are both required for the nicking reaction (Scheiffele *et al*., 1995; Stachel *et al*., 1987; Yanofsky *et al*., 1986). *In vitro*, VirD2 alone nicks ss oligonucleotides bearing the T-DNA border sequence (Jasper *et al*., 1994; Pansegrau *et al*., 1993a). In the presence of an excess of cleavage products, VirD2 can also catalyze the reverse reaction, joining two pieces of ssDNA (Pansegrau *et al*., 1993a). Notably, VirD2 catalyzes the cleavage of the IncP *oriT* in an ss oligonucleotide, but TraI, the IncP homolog of VirD2, cannot cleave a ss T-DNA right border (Pansegrau *et al*., 1993a). The ability to recognize heterologous *nic* sites may reflect a role for VirD2 in initiating the integration of the T-strand to the 3′ side of an ss nick in plant DNA. VirD2 may have evolved to tolerate more variability in the sequences to which it will bind to facilitate T-strand ligation into non-homologous DNA. When *nic* sites are presented in supercoiled double-stranded (ds) plasmids, VirD1 is essential for VirD2 nicking. The VirD1/D2 complex is unable to cleave its cognate *nic* site presented in a relaxed ds circle or a linear double strand.

IncP relaxases have three conserved motifs at their N-termini (Ilyina and Koonin, 1992; Pansegrau *et al*., 1994). Motif I contains a tyrosine that forms a phosphodiester bond between its aromatic hydroxyl group and the 5′ phosphoryl group of the DNA during cleavage (Pansegrau *et al*., 1993b). This tyrosine, at position 29 in VirD2 and position 22 in TraI, cannot be altered without abolishing nicking activity (Vogel and Das, 1992). Motif III is the most highly conserved, and contains two histidines necessary for cleavage that may activate the tyrosine by co-ordinate binding of Mg2+ (Vogel *et al*., 1995). Motif II may recognize a sequence 3′ to the *nic*. Critical amino acids for these functions have been identified in TraI by mutagenesis, and are predicted in VirD2 based on the presence of identical or similar residues within equivalent context (Pansegrau *et al*., 1994). As with *nic* sequences, the similarity among the molecular reactions that cleave *nic* sites and the proteins that mediate these reactions strongly suggests that these systems share a common ancestor.

ssDNA binding proteins (Table 1, item 9)

Transfer of nucleic acids through cell membranes is essential in all living organisms (Citovsky and Zambryski, 1993). Cellular physiology of eukaryotes is dependent on the nuclear import/export of RNA. Pathogenesis, especially viral, often requires genome transport into the nucleus or from cell to cell. During transport, the nucleic acid is potentially a target for nucleolytic degradation. In addition, transported nucleic acids must be efficiently targeted to their sites of action. These functions, protection and localization, are largely provided by proteins associated with nucleic acids (Citovsky and Zambryski, 1993). Identification of the *Agrobacterium* protein VirE2 as a sequence non-specific, ss DNA binding (SSB) protein not only furthered our understanding of *Agrobacterium*-mediated genetic transformation of plants, but also provoked insights in two areas – nuclear import in plant cells and plant virus spread from cell-to-cell.

VirE2 is a *vir*-inducible, ss nucleic acid binding protein (Christie *et al*., 1988; Citovsky *et al*., 1988; Das, 1988; Gietl *et al*., 1987). *In vitro*, VirE2 binds ss DNA regardless of sequence; the binding is strong and co-operative suggesting that T-strands are fully coated withVirE2 (Citovsky *et al*., 1989; Sen *et al*., 1989). VirE2:ssDNA complexes are resistant to 3′ or 5′ exonucleases, as well as endonucleases (Citovsky *et al*., 1989; Sen *et al*., 1989). VirE2:ssDNA complexes formed *in vitro* are unfolded and less than 2 nm in diameter (Citovsky *et al*., 1989), but under some conditions this complex adopts a coiled, telephone cord-like conformation (Citovsky *et al*., 1997).

Binding of VirE2 to T-strand was originally proposed to occur *in bacterium* prior to export (Christie *et al*., 1988; Zupan and Zambryski, 1997). Alternatively, it has been proposed that VirE2 and the VirD2:T-strand are exported independently from the bacterium and formation of the T-complex is completed in the plant cell cytoplasm (Binns *et al*., 1995; Gelvin, 1998; Lee *et al*., 1999; Sundberg *et al*., 1996). That VirE1 physically interacts with VirE2 suggests

the binding of VirE2 to the T-strand is regulated *in vivo* but does not resolve the question of where or when this occurs (Deng *et al.*, 1999; Sundberg and Ream, 1999; Sundberg *et al.*, 1996). In either case, once bound to the T-strand, VirE2 provides protection from nucleolytic degradation. If T-strand is transported as a nucleoprotein complex, a specific conformation, maintained by VirE2, may well be a prerequisite for transit through transmembrane channels that mediate transfer. Before discussing targeting functions of VirE2, we highlight how these results impacted on other research in our laboratory.

The requirement for an SSB in the transfer of nucleic acids during pathogenesis by *Agrobacterium* directly stimulated the hypothesis that a similar activity might be involved in the cell-to-cell spread of plant viruses. Movement of plant viruses from infected cells to healthy cells was long presumed to take place through plasmodesmata (Esau, 1948), natural plant intercellular connections. The estimated size exclusion limit (SEL) of plasmodesmata (0.9–1.0 nm Stokes' radius), however, was far below the size of whole virus particles (12–80 nm) or free viral genomes with irregular, folded structures and Stokes' radii of at least 10 nm. Therefore, to exploit plasmodesmata, plant viruses must increase the plasmodesmata SEL or synthesize a transfer intermediate compatible with transport through plasmodesmata (reviewed in Ghoshroy *et al.*, 1997). Thus, an ss DNA:SSB complex was predicted to serve as the transfer intermediate in movement of viral nucleic acids through plasmodesmata as well as transfer of T-strand from bacterium to plant cell.

Genetic evidence suggested that viral-encoded proteins, termed movement protein (MPs), mediated cell-to-cell movement (reviewed in Carrington *et al.*, 1996; Citovsky, 1999; Mushegian and Koonin, 1993). The first indication of the mechanism behind this activity derived from studies of P30, the MP of tobacco mosaic virus (TMV), a positive sense RNA virus. Transgenic tobacco expressing P30 allowed the diffusion of fluorescently labeled, microinjected 10 kDa dextrans from the injected cell to adjacent cells while the same tracer remained confined to the injected cell in control plants (Wolf *et al.*, 1989). Thus, the SEL of plasmodesmata in transgenic plants had been increased to approximately 3 nm (the Stoke's radius of 10 kDa dextran). Plasmodesmata with these dimensions, however, would still be unable to traffic TMV RNA or viral particles.

Using a biochemical approach, Citovsky *et al.* (1990) demonstrated that P30 binds ss nucleic acids. The binding is strong, co-operative and sequence non-specific. P30:ss nucleic acid complexes are less than 2 nm in diameter, compatible with the P30-induced increase in the SEL of plasmodesmata (Citovsky *et al.*, 1990; Citovsky *et al.*, 1992a). The *in vivo* and *in vitro* activities of P30 generate a very specific model for the spread of plant viruses from cell to cell. After infection and replication of the TMV genome, P30 sequesters a portion of the RNA for MP-mediated transport to adjacent cells while the remainder is encapsidated for spread by vectors to other plants. P30 expression during infection of a leaf is transient, temporally partitioning the replicated genomes between nucleoprotein complexes for cell-to-cell spread and viral particles for transfer to new hosts. After transport into a neighboring cell, replication, cell-to-cell movement and encapsidation are recapitulated.

Since the initial characterization of P30 as an ss nucleic acid binding protein, SSB activity has been demonstrated for many plant viral MPs (Table 2) (reviewed in Carrington *et al.*, 1996; Lazarowitz and Beachy, 1999; Mushegian and Koonin, 1993). The fact that most MPs have SSB activity suggests that transport through plasmodesmata of nucleoprotein intermediates is a common mechanism for cell-to-cell spread of many plant viruses; this simple model was provoked by our studies of VirE2 and the formation of a transferable T-complex by *Agrobacterium*.

Plant nuclear localization signals (Table 1, item 10)

Integration of the T-strand requires that the T-complex be imported into the plant cell nucleus, a tightly regulated process in which proteins larger than 40–60 kDa must possess a nuclear localization signal (NLS) to mediate their import through the nuclear pore (Goerlich, 1997; Melchior and Gerace, 1995). As the T-strand is presumed to be completely coated with proteins, the signals that target T-complex to the nucleus most likely reside in its associated proteins, VirD2 and VirE2 (Zambryski, 1992). Therefore, in the evolution of the *vir*-system to exploit a eukaryotic host, these prokaryotic proteins acquired motifs that function as NLSs. Nuclear targeting of VirD2 and VirE2 were among the first studies of nuclear import in plants.

Nuclear localization of VirD2. Sequence analysis of VirD2 revealed a sequence homologous to the bipartite type of NLS (Howard *et al.*, 1992). The nuclear localizing function of this sequence was confirmed by expressing a VirD2:-GUS fusion in tobacco protoplasts (Howard *et al.*, 1992) and by immunolocalization (Tinland *et al.*, 1992). That *Agrobacterium* was severely reduced in tumorigenicity when the two stretches of basic amino acids in the VirD2 bipartite NLS were deleted validated the biological relevance of the NLS-like sequence (Shurvinton *et al.*, 1992).

VirE2 NLSs (discussed below) are present in vast excess over the single VirD2 NLS. This raises the question of whether the NLS of VirD2 is superfluous or has a unique role in the import of T-complex. The fact that *Agrobacterium* bearing an NLS-deleted VirD2 was attenuated in virulence suggests the latter. Possibly, VirD2 ensures that the 5′ end of the T-strand enters the nucleus

Table 2. Representative plant pathogen proteins with single-strand nucleic acid binding activity*

Pathogen	Protein	Nucleic acid binding activity and function	References
Agrobacterium tumefaciens	VirE2	ssDNA; protection and nuclear import	(Christie *et al.*, 1988; Citovsky *et al.*, 1988; Citovsky *et al.*, 1989; Citovsky *et al.*, 1992; Das, 1988; Sen *et al.*, 1989)
Tobacco mosaic virus (TMV)	P30	ssRNA, ssDNA; cell-to-cell movement	(Citovsky *et al.*, 1990; Citovsky *et al.*, 1992a; Waigmann *et al.*, 1994)
Cauliflower mosaic virus (CaMV)	P1	ssRNA, ssDNA; cell-to-cell movement	(Citovsky *et al.*, 1991; Thomas and Maule, 1995)
Squash leaf curl virus (SqLCV)	NSP (BV1, BR1) MPB (BC1, BL1)	ssDNA; nuclear shuttle, cell-to-cell movement	(Pascal *et al.,* 1994; Sanderfoot and Lazarowitz, 1995; Sanderfoot *et al.*, 1996)
Cucumber mosaic virus (CMV)	3a	ssRNA, ssDNA; cell-to-cell movement	(Ding *et al.*, 1995; Li and Palukaitis, 1996; Vaquero *et al.*, 1997)
Barley stripe mosaic virus (BSMV)	βb	ssRNA, dsRNA	(Donald *et al.*, 1997)
Potato leafroll virus (PLRV)	pr17	ssRNA, ssDNA	(Tacke *et al.*, 1991)
Potato virus Y (PVY)	HC-Pro	ssRNA	(Maia and Bernardi, 1996)

*Additional movement proteins with SSB activity are described in Mushegian and Koonin (1993), Carrington *et al.* (1996), and Lazarowitz and Beachy (1999).

first, avoiding entry in an awkward 'elbow' conformation. Initiation at the 5′ end may be a common feature of the translocation of ss nucleic acids across the nuclear pore in either direction. For example, export of 75s rRNA (Balbioni Rings) from the nuclei of salivary gland cells in *Chironomus tentans* initiates at the 5′ end (Mehlin *et al.*, 1992).

VirD2 was exploited in yeast two-hybrid analysis to identify an *Arabidopsis* protein of the karyopherin-α family (AtKAPα) (Ballas and Citovsky, 1997). AtKAPα rescued a yeast mutant defective in nuclear import and mediated nuclear import of VirD2 in permeabilized yeast cells. The interaction between VirD2 and AtKAPα provides strong evidence that *Agrobacterium* has co-opted the eukaryotic process of nuclear import to assist in the efficient genetic transformation of plant cells.

Nuclear localization of VirE2. VirE2, the most abundant protein component of the T-complex, contains two bipartite NLSs. Both NLSs were required for maximum accumulation of a VirE2:GUS fusion protein in the nucleus (Citovsky *et al.*, 1992b). The T-strand from the nopaline strain of *Agrobacterium* requires 600 monomers of VirE2 to coat it completely (Zambryski, 1992). Therefore, VirE2 provides NLSs along the entire length of the T-complex, potentially facilitating uninterrupted nuclear uptake of T-complex.

VirE2-mediated nuclear import of ssDNA was assayed directly by microinjection of complexes formed *in vitro* from fluorescently labeled DNA and purified VirE2 into stamen hair cells of the flowering plant *Tradescantia virginiana* (Zupan *et al.*, 1996). Fluorescent DNA accumulated in the nucleus specifically when microinjected as a complex with VirE2. ssDNA microinjected alone remained cytoplasmic. These data suggest that T-strand is imported into the nucleus by a protein import pathway via its association with VirE2. Other data have been interpreted to suggest that VirE2 does not provide nuclear-targeting but only protects T-strand from nucleolytic degradation inside the plant cell (Rossi *et al.*, 1996); however, the entire *virE2* gene was deleted, simultaneously abolishing both protective and nuclear localizing functions.

T-complex nuclear import also reveals novel features of this process. First, accumulation of VirD2 and VirE2 GUS-fusions in the nuclei of leaf and immature root epidermal cells, but not in mature root epidermal cells, suggests nuclear import may be developmentally regulated. Cell-type specific NLS-binding proteins could result in specific gene expression by admitting different subsets of transcription factors into the nucleus (reviewed in Whiteside and Goodbourn, 1993). Secondly, plants may tolerate greater variability in the primary sequence of bipartite NLSs. While the NLS of VirD2 conforms to the consensus 'animal' bipartite sequence, the NLSs of VirE2 are less homologous. Both of these proteins are imported into plant cell nuclei but only VirD2 is imported into the nuclei of animal cells (Guralnick *et al.*, 1996). When either NLS of VirE2 was altered to conform precisely to the 'animal' bipartite signal, the protein accumulated in animal cell nuclei and mediated nuclear import of ss nucleic acid (Guralnick *et al.*, 1996).

Integration (Table 1, item 11)

In the final step of T-DNA transfer, the incoming ssDNA of the T-complex is stably integrated into a plant chromosome (Gheysen *et al.*, 1991). Integration may induce small

deletions, less than 100 bp, of plant DNA at the insertion site. In addition, integration also generates 'filler' DNA, less than 50 bp homologous to nearby plant DNA, at the T-DNA-plant junction. Integration of the 5′ end of the T-strand is relatively precise often occurring at the penultimate 5′ nucleotide bound to VirD2 (Tinland *et al.*, 1995). Some mutations in VirD2 result in large deletions at the 5′ end suggesting that VirD2 mediates the precision of integration at this end. At the 3′ end, integration usually includes most of the left border region, although larger deletions are more common at this end. Deletions of up to 1000 bp at the 3′ end of the T-strand transferred from a *virE2*⁻ strain suggest that VirE2 plays a significant role in protecting the T-strand from nucleolytic degradation (Rossi *et al.*, 1996).

The mechanics of integration remain largely unknown. After nuclear import, the T-strand is likely made ds with the concomitant displacement of VirE2. Conversion to a ds form is supported by transient expression of reporter genes in the T-DNA, extrachromosomal homologous recombination of T-DNA prior to integration, and the complex pattern of multiple T-DNAs at a single insertion site (discussed in De Buck *et al.*, 1999).

Given that the T-strand does not encode enzymes that catalyze integration, these functions must be mediated by host cell factors. As not more than 5 (usually 0–3) bp of homology have been found between either end of the T-DNA and the plant sequences at the insertion site, most models usually propose illegitimate recombination as the model for T-strand integration (De Buck *et al.*, 1999). Double-strand break repair via non-homologous end-joining (Britt, 1999) of the ds T-strand is consistent with many of the products observed as a result of T-DNA integration. The precision of T-DNA borders at the 5′ end of the T-strand suggested that VirD2 initiates integration by ligating the 5′ end to an exposed 3′-OH in plant DNA. An alternative model proposes that the T-strand remains ss, and integration initiates by docking of the 3′ end of the T-strand via 2–5 bases of microhomology (Tinland *et al.*, 1995). The latter model, however, does not explain the pattern of integration for tandem arrays of multiple T-DNAs. In either case, both 5′ and 3′ integration are likely to be assisted by plant machinery for DNA metabolism; DNA replication and repair type enzymes are prime candidates. It will be interesting to determine whether VirD2 and VirE2 directly interact with structural or enzymatic components of plant chromatin or DNA metabolism.

Type IV secretion systems (Table 1, item 12)

Systems that secrete various substrates through the bacterial envelope are currently classified into four types. Type I, typified by *Escherichia coli* hemolysin export, is sec-independent and requires 3–4 accessory proteins (Fath and Kolter, 1993). In the type II system, typified by pullulanase export in *Klebsiella oxytoca*, the substrate is exported into the periplasm by the *sec*-dependent general secretory pathway (GSP) then secreted across the outer membrane via a specialized terminal branch of the GSP (Pugsley, 1993). Type III secretion (Hobbs and Mattick, 1993), typified by Yop export in the human pathogen *Yersinia pestis* (Cornelis and Wolf-Watz, 1997), is sec-independent, translocates protein substrates from the bacterial cytoplasm into the eukaryotic host cytoplasm, and requires more than 20 accessory proteins. Type III systems also include bacterial flagellar assembly proteins which 'secrete' flagellar components to the exterior of the cell.

Type IV secretion systems comprise a growing family of multiprotein complexes that span the bacterial envelope (Salmond, 1994). Originally, the type IV family included the *virB* operon and systems for conjugal DNA transfer. Homologs of six *virB* genes were then found in the *ptl* operon of *Bordetella pertussis*, which encodes a transporter that exports pertussis toxin (reviewed in Lessl and Lanka, 1994; Winans *et al.*, 1996). Subsequently, type IV systems essential for virulence have been identified in human pathogens (discussed below), and mediate transfer of proteinaceous factors, such as CagA (Odenbreit *et al.* 2000; Stein *et al.* 2000), that subvert host defense mechanisms. The export of proteins suggests type IV secretion systems evolved from a protein exporter system. The *Agrobacterium* VirB transporter as well as those for bacterial conjugation may incidentally transfer DNA by virtue of its forming a nucleoprotein complex with proteins targeted for export, exemplified by the T-complex.

Recent advances: bacterial secretion and pathogenesis

Structure and function of the T-transporter

Over the past 5 years, much of the research on *Agrobacterium*-mediated gene transfer has focused on the *vir*-specific type IV secretion system, the T-complex transporter (T-transporter). This apparatus is assembled from 11 proteins encoded by the *virB* operon, and VirD4. The T-transporter facilitates transfer of the T-complex to plant or yeast cells and mediates conjugal transfer of the non-self transmissible, but mobilizable, plasmid RSF1010 (an IncQ plasmid) to *Agrobacterium*. It can also transfer proteins, such as VirE2 and VirF, to plant cells (reviewed in Christie, 1997; Zupan *et al.*, 1998).

While the mechanics of T-transporter function are not known, the basic architecture of its structural components is emerging. Assembly is most likely preceded by hydrolysis of the peptidoglycan layer by VirB1 (Figure 2a) (Baron *et al.*, 1997a). This activity is essential for the mobilization

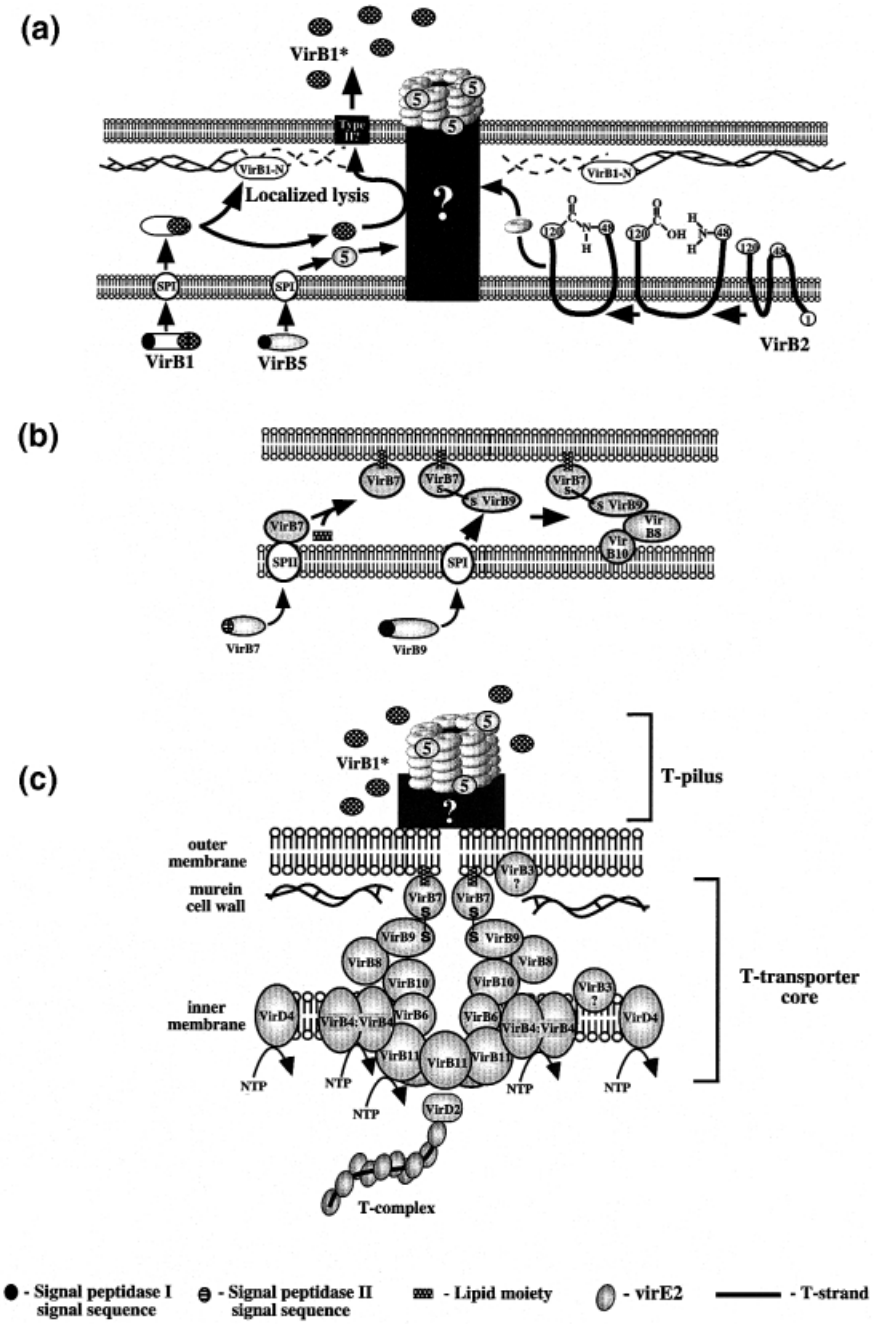

Figure 2. Assembly of the *Agrobacterium tumefaciens* T-complex transporter.
(a) Assembly of the T-pilus. VirB1 is processed into two domains following SPI mediated transport into the periplasm. The N-terminal portion (VirB1-N, white oval) may be involved indirectly in pilus formation (as well as assembly of the transporter core) by inducing localized lysis of the peptidoglycan layer. The C-terminal VirB1* (cross-hatched oval) may chaperone pilus components VirB2 and VirB5 to the exterior of the cell. VirB2 (major) and VirB5 (minor) pilus components are shown as donut-shaped and gray ovals, respectively. VirB2 processing is described in the text. VirB2 and VirB5 are mobilized to the cell surface by an unknown mechanism that likely requires additional VirB proteins. The black box indicates that it is not known how the T-pilus is anchored in the bacterial envelope.
(b) Assembly of the T-complex transporter core begins with the lipid-modification of VirB7 resulting in anchoring in the outer membrane. VirB7 forms a heteromultimer with VirB9 which promotes the stability of many other VirB proteins, possibly through interactions with VirB8 and VirB10 (Das and Xie, 2000).
(c) The T-complex transporter is suggested to comprise a pilus and channel 'core' (see text). Whether the pilus and core are coupled either physically or functionally is not known (black box).

of VirB2 and VirB5 to the cell surface to form the T-pilus, the best documented structural feature of the transporter (Figure 2a). Interaction and subcellular localization studies suggest at least some of the remainder of the VirB/VirD4

proteins assemble into a multimeric complex that spans the bacterial envelope (Figure 2b). This hypothetical 'core' of the T-transporter likely translocates substrates out of the bacterium. Energy to drive assembly and translocation is potentially provided by the three ATPases (VirB4, VirB11 and VirD4) associated with the T-transporter (Figure 2c) (reviewed in Christie, 1997; Zupan *et al.*, 1998). Increasingly, non-*vir* functions are also being identified as essential for transporter assembly. Below, we discuss each of the known components of the T-transporter.

VirB1 is bifunctional

Illustrations depicting large, multicomponent, membrane-spanning complexes in bacteria usually neglect to represent the peptidoglycan layer (Dijkstra and Keck, 1996). This omission reflects the lack of information on how penetration of this layer is achieved during complex assembly. The size limit for diffusion through naturally occurring channels in the peptidoglycan layer is only ≈ 50 kDa (Dijkstra and Keck, 1996), too small to allow the assembly of a multiprotein apparatus large enough to traffic nucleoprotein complexes. Work on VirB1 provided some of the first evidence that secretion systems may include dedicated factors that modify the bacterial cell wall to accommodate their assembly. Sequence comparisons revealed similarities among chicken egg white lysozyme and the N-terminus of VirB1, as well as proteins involved in conjugal DNA transfer, invasion by the human pathogens *Salmonella enterica* and *Shigella* spp. and others (Table 3) (Dijkstra and Keck, 1996; Lehnherr *et al.*, 1998; Mushegian *et al.*, 1996). Based on lysozyme homology, these proteins are predicted to possess glycosidase activity, and indeed they share homology with lytic transglycosylases across a region that forms the catalytic site (Table 3). Although VirB1 glycosidase activity has not been demonstrated directly *in vitro*, agrobacteria, carrying VirB1 mutations in two residues predicted to be critical for hydrolysis of glycosidic bonds were severely attenuated in virulence (Mushegian *et al.*, 1996).

VirB1 is processed to release its C-terminal third, VirB1*, which is then secreted (Baron *et al.*, 1997a). Approximately half of VirB1* remains loosely associated with the exterior of *Agrobacterium* cells, and the rest can be recovered from culture supernatants (Baron *et al.*, 1997a). Processing and secretion of VirB1* are not coupled, are independent of the Ti-plasmid, but do require the signal peptide (Llosa *et al.* 2000). Thus, factors that mediate processing of VirB1 and secretion of VirB1* are localized in the periplasm or outer membrane and are not subject to *vir*-regulation. VirB1 is processed to VirB1* in other Rhizobiaceae, but secretion is specific to *Agrobacterium* (Weininger, Domke and Baron, personal communication). These observations suggest that VirB1* is synthesized and secreted for a specific role, most likely at the cell surface, during T-transporter assembly or function. A bifunctional VirB1 is supported by partial complementation (tumor and T-pilus assays) of a *virB1* deletion strain with constructs expressing either the N-terminal lysozyme-homologous region or the C-terminal VirB1* (Llosa *et al.* 2000; Weininger, Domke and Baron, personal communication). Thus, each domain of VirB1 probably has its own role in tumorigenesis.

As VirB1 is the first product of the polycistronic transcript of the *virB* operon, it is likely to have an early role. Its loose exterior location suggests that VirB1* may play a non-structural role mediating pilus formation, such as chaperone activity for VirB2 during transport to the cell exterior. Alternatively, association of VirB1* with VirB9 (Baron *et al.*, 1997a) suggests that it may function in the assembly of the T-transporter 'core', perhaps before VirB1* is secreted to the exterior of the cell. Finally, association of VirB1* with the exterior of *Agrobacterium* suggests that it may be available to interact with the plant cell surface (Figure 2a).

T-pilus

The only documented structural component of the T-transporter is the T-pilus, a long, flexible, filamentous appendage observed on the surface of *vir*-induced

Table 3. Selected proteins with lysozyme or transglycosylase homology

Protein	Organism or plasmid	Function	Reference
VirB1	*A. tumefaciens*	Transporter assembly	(Baron *et al.*, 1997a; Dijkstra and Keck, 1996; Mushegian *et al.*, 1996)
VirB1	*Brucella suis*	Survival; intracellular multiplication	(O'Callaghan *et al.*, 1999)
TraN	IncP plasmid	Conjugation	(Lessl *et al.*, 1992)
TraL	IncN plasmid	Conjugation	(Mushegian *et al.*, 1996; Pohlman *et al.*, 1994)
ORF19/ORF169	IncFII R1 plasmid	Conjugation	(Graus *et al.*, 1990; Loh *et al.*, 1989)
Slt70	*E. coli*	Peptidoglycan degradation	(Holtje *et al.*, 1975; Thunnissen *et al.*, 1994)
IpgF	*Shigella* spp.	Invasion	(Allaoui *et al.*, 1993)
IagB	*Salmonella enterica*	Invasion	(Miras *et al.*, 1995)
Pm404	*Proteus mirabilis*	?	(Dijkstra and Keck, 1996)

Agrobacterium cells (Fullner *et al.*, 1996). Formation of T-pili is dependent on expression of VirB proteins and is correlated with VirB-mediated transfer of T-complex to plant cells and the transfer of IncQ plasmids between bacteria (Fullner, 1998).

The F-pilus of the F-incompatibility system of *E. coli* is the best characterized component of any type IV transporter (Firth *et al.*, 1996). This pilus mediates cell surface contact between donor and recipient, a prerequisite for conjugal DNA transfer. F-pili have been observed to shorten and 'retract.' Presumably, retraction brings the cell surfaces of the donor and recipient into close contact to facilitate transfer of F-plasmid.

The inside diameter of the F-pilus (2 nm) (Silverman, 1997) is barely compatible with the transfer of nucleoprotein complexes through the lumen. The diameter of the nucleoprotein T-complex is < 2 nm (Citovsky *et al.*, 1989). Although the interior dimensions of the *Agrobacterium* virulence pilus have not been reported, the exterior dimensions (10 nm) (Lai and Kado, 1998) are comparable to the F-pilus (8 nm) (Silverman, 1997). As the T-pilus is extremely thin and the T-strand is likely to be transferred as a nucleoprotein complex, it is difficult to imagine how the T-pilus lumen serves as a conduit for delivery of an elongated T-complex into the host without some additional features. Either a conformational change in the pilus structure itself and/or the widening of the transmembrane channel may diminish the physical constraints to egress of the T-complex. Whether the pilus serves as the conduit for T-complex transport, as well as the host cell tether, remains unresolved. If the pilus serves only as a tether, the T-complex may move directly through the VirB transmembrane channel into the plant cell by an unknown mechanism. Dürrenberger *et al.* (1991) suggest that conjugating bacteria become tightly juxtaposed along their length to form patches of intimate contact. If the tether and conduit are separated functionally, they may not be linked physically. The latter scenario provides two opportunities for interaction between the host cell surface components and the bacteria, i.e. interactions via the pilus and interactions via the channel.

Virulence pili may also have a regulatory role in T-transporter function. While attachment of *Agrobacterium* is obviously critical for the genetic transformation of plant cells, attachment is chromosomally, not Ti-plasmid, encoded (Matthysse and Wagner, 1994). Furthermore, expression of the *vir*-specific type IV transporter components can be induced by soluble plant factors in the absence of plant cell contact *per se.* T-complex, however, is not detected in *vir*-induced culture supernatants; thus, the T-transporter may not be functional without a plant host cell. Type III secretion systems require host cell contact for transporter assembly and function (Cornelis and Wolf-Watz, 1997). Perception and transduction of a contact signal by the T-pilus may trigger transporter activity, either by assembling transporter components at the site of closest proximity to the recipient cell or by inducing activity in assembled transporters specifically in the region of contact.

T-pilus assembly. Two Vir proteins have been identified in T-pili preparations. The major component, VirB2, is translated as a 12.3 kDa protein that is quickly processed to a 7.2 kDa protein associated with the inner membrane (Jones *et al.*, 1996; Lai and Kado, 1998). The VirB2 homolog, TraA, is the F-pilus subunit and is similarly processed from a 12.3 kDa propilin to a 6.2 kDa inner membrane protein (Firth *et al.*, 1996). In the presence of recipient cells, TraA is mobilized by an unknown mechanism to the surface of the donor cell where it polymerizes to form the pilus. In contrast, *Vir*-induced cultures of *Agrobacterium* assemble T-pili without host plant cells (Lai and Kado, 1998; Schmidt-Eisenlohr *et al.*, 1999a). Strains of *Agrobacterium* with deletions of *virB3*, *virB4*, *virB5*, *virB9* or *virB10* produce VirB2, but the processed form accumulates inside rather than on the exterior of the cell, and pili are not observed (Lai and Kado, 1998).

The leader peptide (47 amino acids) of VirB2 propilin is cleaved during insertion into the inner membrane where it accumulates prior to T-pilus assembly (Eisenbrandt *et al.*, 1999). Pilin subunits in other systems undergo a variety of further post-translational modifications. TraA of the F-pilus is acetylated at the C-terminus (Moore *et al.*, 1993), and the subunit of type IV adhesive pili in *Neisseria meningitidis* is glycosylated (Virji *et al.*, 1993). VirB2, as well as its IncP homolog TrbC, forms an intramolecular bond between its N- and C-termini producing a cyclic polypeptide, a rare reaction in prokaryotes (Eisenbrandt *et al.*, 1999). Prior to cyclization, TrbC undergoes two additional proteolytic reactions following leader peptide removal; the final reaction requires TraF and removes four amino acids at the C-terminus. In the absence of TraF, TrbC is not cyclized; cyclization may have specific sequence requirements necessitating prior processing by TraF. The Ti-plasmid does not encode a TraF homolog and VirB2 does not undergo proteolytic processing subsequent to removal of the signal peptide (Figure 2a).

VirB2 is cyclized in the absence of other Ti-plasmid genes but little is known about this reaction (Eisenbrandt *et al.*, 1999). Membrane topology studies predict that both the N- and C-termini of VirB2 protrude into the periplasm after insertion into the inner membrane (Eisenbrandt *et al.*, 1999). This conformation may facilitate formation of the intramolecular bond by bringing the polypeptide termini into close contact (Figure 2a). Either cyclization is autocatalytic or the putative cyclase represents a novel unidentified chromosomal factor required for virulence.

In addition to VirB2, VirB5 is found as a minor component in T-pili preparations (Schmidt-Eisenlohr *et al.*,

1999a). TraC, the IncN VirB5 homolog, also associates with an exocellular polymeric structure that may be the pilus of pKM101 (IncN) (Schmidt-Eisenlohr *et al.*, 1999b) thus suggesting that VirB5 homologs may function as auxiliary structural proteins in pili of type IV secretion systems (Figure 2a). In contrast to VirB2, cellular levels of VirB5 were strongly correlated with the abundance of other *Vir* proteins, indicating its stabilization by protein–protein interactions (Schmidt-Eisenlohr *et al.*, 1999a). Thus, additional VirB components, as well as VirB2 and VirB5, are required for T-pilus formation. Isolated pili have terminal knobs, but it is unknown whether these knobs reside at the pilus tip or base anchoring the pilus in the bacterial envelope (Schmidt-Eisenlohr *et al.*, 1999a). By analogy to pap–pili adhesins, the knob may be a distal feature of the T-pilus that mediates interaction with the plant cell.

Assembly of the T pilus also involves VirB6 (Fullner *et al.*, 1996). Based on its predicted extreme hydrophobic character, VirB6 was suggested to form a pore in the inner membrane (Christie, 1997; Das and Xie, 1998) that might function in egress of the T-complex. Although cellular levels of most VirB proteins were unaffected by in-frame deletion of *virB6*, pili were not formed (Hapfelmeier, Domke, Zambryski and Baron, personal communication). Pili were restored by *trans*-complementation with *virB6*, suggesting that VirB6 may function in pilus assembly and not directly in the translocation of the T-complex.

Assembly of a T-complex transporter 'core'

A membrane spanning conduit is generally assumed to represent the channel 'core' for T-complex export. Insights into the composition of this core, its assembly or its function require determination of the specific protein–protein interactions.

VirB7, VirB8, VirB9 and VirB10. The best documented interaction for components of the T-transporter is between the outer membrane lipoprotein VirB7 and periplasmic VirB9 (Anderson *et al.*, 1996; Baron *et al.*, 1997b; Das *et al.*, 1997; Fernandez *et al.*, 1996a; Spudich *et al.*, 1996). Strains carrying mutations in VirB7 or VirB9 that disrupt formation of the heteromultimer accumulated significantly lower cellular levels of VirB4, VirB5, VirB8, VirB10 and VirB11 (Fernandez *et al.*, 1996b). In the absence of the VirB7:VirB9 heterodimer, these proteins may be degraded. Thus, the VirB7:VirB9 heterodimer may play a critical role initiating or stabilizing transporter assembly through physical interactions (Figure 2b).

VirB10 forms high molecular weight complexes, dependent on the VirB7:VirB9 heterodimer (Beaupré *et al.*, 1997; Ward *et al.*, 1990). Two-hybrid analysis in yeast indicates VirB10 interacts physically with VirB9 as well as VirB8 (Das and Xie, 2000), although these proteins were not identified in the VirB10 complexes in *Agrobacterium* (Beaupré *et al.*, 1997; Ward *et al.*, 1990). Alteration of a single amino acid in VirB8 disrupted the two-hybrid interaction with VirB9 and caused avirulence suggesting the VirB8:VirB9 interaction is essential. Possibly, VirB8:VirB9 association in the periplasm forms links with VirB10 in the inner membrane and with VirB7 in the outer membrane (Figure 2c).

The ATPases: VirB4, VirB11 and VirD4. The T-transporter includes three proteins (VirB4, VirB11 and VirD4) with homology to ATPases. These proteins are presumed to provide the energy that drives either transporter assembly, T-complex translocation, or both. Strains carrying VirB4 mutants with defects in the Walker A nucleotide binding motif are avirulent (Christie *et al.*, 1989; Fullner *et al.*, 1994) and exert a dominant negative effect when co-expressed with wild-type VirB4 (Berger and Christie, 1993). Trans-dominance suggests VirB4 is incorporated into a multimer. VirB4 also mediated dimerization of the λ c1 repressor protein and conferred immunity to λ infection (Dang *et al.*, 1999). As the VirB4 Walker A motif mutants also conferred λ immunity, ATP binding is dispensable for dimerization. However, dimerization may be essential for ATPase activity as occurs in ATPase subunits associated with a variety of membrane transport systems (Davidson *et al.*, 1996; Nikaido *et al.*, 1997).

Co-synthesis, specifically of VirB3, VirB4, VirB7, VirB8, VirB9 and VirB10, in agrobacterial recipient cells significantly stimulates VirB-mediated conjugation of the non-self transmissible IncQ plasmid RSF1010 (Bohné *et al.*, 1998). The increase in conjugation efficiency, proposed to result from the assembly of these VirB proteins into a transmembrane structure, may provide an assay for testing hypotheses regarding the assembly and function of individual VirB proteins. For example, recipients expressing either wild-type VirB4 or Walker A box mutants exhibit the same level of enhanced conjugation efficiency. Potentially, VirB4 homomultimers are required for the assembly of a complex including additional VirB proteins, and this structure can assemble in the absence of ATP-binding by VirB4 (Dang *et al.*, 1999). In this scenario, VirB4 may act as an essential component of the scaffolding for transporter assembly. In contrast, the donor requirement for a wild-type VirB4 suggests that ATP-binding confers the T-transporter with 'one-way' activity to export DNA (Figure 2c).

The homology between VirB4 and VirB3 and the IncF proteins TraL and TraC, respectively, suggests that these *Agrobacterium* proteins have a role in pilus assembly (Jones *et al.*, 1994). The IncF proteins are essential for pilus formation but are not pilus structural components. While a connection to T-pilus assembly has not been established for either VirB4 or VirB3, they may physically interact. In a *virB4* deletion strain, cellular levels of VirB3 are reduced,

and VirB3 is localized to the inner membrane rather than both inner and outer membranes as in wild-type strains (Jones *et al.*, 1994). VirB3 specific localization, mediated by VirB4, may promote mobilization of VirB2 to the exterior of the cell.

VirB11 is a member of the PulE superfamily and is the most widespread of the VirB proteins (Krause *et al.*, 2000). In addition to the type IV secretion systems, homologs are found in fimbrial genes from *Pseudomonas aeruginosa* and pathogenic *Neisseria*, the comG operon involved in *Bacillus subtilis* competence, and the pullulunase secretion system of *Klebsiella oxytoca*. PulE homologs are cytoplasmic or weakly associated with the cytoplasmic face of the inner membrane. Two members of this family, TrbB of the IncPα plasmid RP4 and HP0525 from the *cag* pathogenicity island of *Helicobacter pylori*, form six membered ring-shaped structures *in vitro* (Krause *et al.* 2000). In the absence of ATP, TrbB formed significantly fewer rings. Other di- and monophosphate nucleotides stabilized the TrbB hexamers as well, indicating that nucleotide binding but not hydrolysis is required. Curiously, HP0525 formed rings even without NTPs. In *Agrobacterium*, mutations in the Walker A box of VirB11 are not transdominant but increase VirB11 association with the cytoplasmic face of the inner membrane (Rashkova *et al.*, 1997). If VirB11 is similar to TrbB, loss of ATP-binding may prevent this class of VirB11 mutants from assembling into hexamers at the inner membrane or from interacting with other VirB components in the transporter. TrbB/HP0525 hexameric rings have an outer diameter of about 12 nm and a central channel approximately 3 nm in diameter. Perhaps the first step in transfer of the T-complex from the bacterial cytoplasm occurs through the channel in a hexameric ring of VirB11 (Figure 2c).

VirD4 homologs are present in almost all type IV secretion systems as well as the F-system (Christie, 1997). All homologs possess a Walker A motif necessary for function and all are integral inner membrane proteins. Mutants of the homologs *traD* (F) and *traG* (RP4, IncP) still produce conjugal pili and attach to recipient cells, but conjugal DNA transfer does not occur (Balzer *et al.*, 1994; Firth *et al.*, 1996). Thus, VirD4 homologs are suggested to mediate introduction of the nucleoprotein complex into the transporter by an energy-dependent mechanism (Cabezon *et al.*, 1997).

New required chromosomal activities

Although the virulence of *Agrobacterium tumefaciens* is largely attributed to the combined activities of the *Vir* proteins, recent work suggests more and varied contributions by chromosomally encoded factors. Attachment was the first step in virulence shown to be mediated by chromosomally encoded products. These *chv* (chromosomal virulence) genes encode proteins that function in synthesis and export of polysaccharides thought to enable recognition and binding of host cell-surface factors (Matthysse and Wagner, 1994). In fact, such polysaccharides may facilitate non-specific 'sticking' to plant cells, as additional data suggest that these proteins contribute to the stability of the bacterial envelope by regulating the periplasmic osmoticum (Swart *et al.*, 1994).

Sequence analysis has identified signal peptidase I and II sites in several VirB proteins (Kuldau *et al.*, 1990; Ward *et al.*, 1988), which implies constitutive secretion pathways are involved in the assembly of the transporter. More specific activities are also indicated. After VirB1 is delivered into the periplasm by the general secretory pathway, processing of VirB1, potentially by a periplasmic protease, releases VirB1* (Baron *et al.*, 1997a). Subsequently, a type II secretion system may transport VirB1* to the exterior of the cell. This would be analogous to the secretion of elastase by *Pseudomonas aeruginosa* (McIver *et al.*, 1995). Elastase is exported into the periplasm by the general secretory pathway where an intramolecular domain, which serves as a chaperone, is cleaved autoproteolytically but remains associated with the elastase. Secretion of the elastase across the outer membrane is mediated by the Xcp apparatus, a type II secretion apparatus required for pathogenicity (McIver *et al.*, 1995).

Intramolecular cyclization, a process required for maturation of VirB2, is an uncommon protein modification; either cyclization occurs autocatalytically or it requires a function encoded on the chromosome (Eisenbrandt *et al.*, 1999). Interactions between VirB proteins and chromosomal factors important for transporter assembly and function are fertile areas for future investigation.

Type IV secretion: a rapidly growing family

The type IV secretion system family is expanding. In addition to conjugal plasmid systems, recently identified homologs occur in animal pathogens and are required for virulence. The list includes *Bordetella pertussis* (whooping cough), *Helicobacter pylori* (gastric ulcers), *Legionella pneumophila* (Legionnaire's disease) and *Rickettsia prowazekii* (epidemic typhus), reviewed in (Christie, 1997; Covacci *et al.*, 1999). As these bacteria represent members of the α, β, ε and γ subgroups of the Proteobacteria, type IV transporters have clearly evolved for a variety of purposes.

Gene clusters in *Brucella suis* (brucellosis) (O'Callaghan *et al.*, 1999) and *L. pneumophila* (Segal *et al.*, 1999) are the most recent additions to the type IV secretion system family. Notably, these organisms possess nearly complete sets of *virB* and *virD4* homologs. *L. pneumophila* contains *virB2-11* and *virD4*, but their specific roles in pathogenesis have not been demonstrated (Segal *et al.*, 1999). *B. suis*

has a gene cluster, also referred to as *virB*, that appears to be a single transcriptional unit and includes homologs of all *Agrobacterium virB* genes in the same order. A twelfth gene in this cluster encodes a protein similar to a mating pair factor from *Enterobacter aerogenes* and adhesin from *Pseudomonas* sp. Strains carrying mutations in either *B. suis virB5* or *virB9* were unable to survive or multiply intracellularly in a variety of cultured animal cells (O'Callaghan *et al.*, 1999). *B. suis* strains with mutations in *virB2*, *virB4* or *virB10* were severely attenuated in rates of intracellular multiplication. Thus, the *virB* region in *Brucella suis* is essential for survival and multiplication in macrophages.

Perspectives

Originally, interest in *Agrobacterium* was sparked by the fact that it caused tumors, and research was expected to provide clues into animal tumor pathogenesis. While this goal was never realized, after nearly 100 years, it is now evident that research in 'Agrobiology' has application to animal pathogenesis. Many bacteria employ a type IV secretion system to transfer conjugal plasmids carrying resistance factors into bacterial recipients and virulence determinants, either DNA or protein, into susceptible plant or animal hosts. Assembly of the *Agrobacterium* T-complex transporter currently serves as the paradigm for type IV secretion systems. The T-transporter transports nucleoprotein complexes as well as protein alone. Of type IV transporters, the T-transporter also recognizes the greatest phylogenetic diversity of hosts, i.e. plants, bacteria and yeast. These attributes confer unparalleled flexibility in experimental design. Given the significance of type IV secretion systems in plant biotechnology and health issues, research on *Agrobacterium*-mediated gene transfer should continue to supply us with insight into a wide range of biological questions.

Acknowledgements

We thank Christian Baron and members of his laboratory for sharing data prior to publication. Our work on *Agrobacterium tumefaciens* was supported by a grant from the National Science Foundation (IBN 9507782) to P. Zambryski, and ongoing research is supported by the Novartis Alliance for Discovery and Innovation.

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