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Pathogen profile

From host recognition to T-DNA integration: the function of bacterial and plant genes in the *Agrobacterium*–plant cell interaction

TZVI TZFIRA AND VITALY CITOVSKY*

Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794-5215, USA

SUMMARY

Agrobacterium tumefaciens and its related species, *A. rhizogenes* and *A. vitis*, are the only known bacterial pathogens which 'genetically invade' host plants and stably integrate part of their genetic material into the host cell genome. Thus, *A. tumefaciens* has evolved as a major tool for plant genetic engineering. Furthermore, this unique process of interkingdom DNA transfer has been utilized as a model system for studies of its underlying biological events, such as intercellular signalling, cell-to-cell DNA transport, protein and DNA nuclear import and integration. To date, numerous bacterial proteins and several plant proteins have been implicated in the *A. tumefaciens*–plant cell interaction. Here, we discuss the molecular interactions among these bacterial and plant factors and their role in the *A. tumefaciens*–plant cell DNA transfer.

Taxonomic relationship: Bacteria; Proteobacteria; alpha subdivision; *Rhizobiaceae* group; *Rhizobiaceae* family; *Agrobacterium* genus.

Microbiological properties: Gram-negative, nonsporing, motile, rod-shaped, soil-borne.

Related species: *A. rhizogenes* (causes root formation in infected plants), *A. vitis* (causes gall formation on grapevines).

Disease symptoms: Formation of neoplastic swellings (galls) on plant roots, crowns, trunks and canes. Galls interfere with water and nutrient flow in the plants, and seriously infected plants suffer from weak, stunted growth and low productivity.

Host range: One of the widest host ranges known among plant pathogens; can potentially attack all dicotyledonous plant species. Also, under controlled conditions (usually in tissue culture), can infect, albeit with lower efficiency, several monocotyledonous species.

Agronomic importance: The disease currently affects plants belonging to the rose family, e.g. apple, pear, peach, cherry, almond, roses, as well as poplar trees (aspen).

Useful web site: http://www.bio.purdue.edu/courses/gelvinweb/gelvin.html

* *Correspondence*: E-mail: vitaly.citovsky@sunysb.edu

INTRODUCTION

Agrobacterium tumefaciens is a soil phytopathogenic bacterium (Fig. 1A) that elicits the crown gall disease in many plant species. The disease is characterized by the formation of large tumours (galls) which typically occur on the plant stem (Fig. 1B), just above the soil level (the plant crown). Although *A. tumefaciens* has a wide host range that includes most of the dicotyledonous plants (de Cleene and de Ley, 1976), the economic importance of the crown gall disease is limited to members of the rose family (Pionnat *et al*., 1999), e.g. rose, apple, pear, peach, cherry and almond.

Unlike other plant pathogenic bacteria which affect the host plant physiology by secreting compounds such as toxins or growth regulators, *A. tumefaciens* and its related pathogenic species, *A. rhizogenes* and *A. vitis*, directly modify the genetic material of their hosts. This genetic modification results from the transfer and integration into the plant genome of a specific DNA segment, termed transferred DNA or T-DNA, from the bacterial Ti (tumour-inducing) plasmid. *In planta* expression of several oncogenic (*onc*) genes encoded by the T-DNA leads to the formation of tumours (Gaudin *et al*., 1994) and the production and secretion of specific amino acid and sugar phosphate derivatives (opines) which are then exclusively utilized by the bacterium as a carbon/nitrogen source. Agrobacteria are usually classified by the subset or type of opines they specify, the most common strains being octopine or nopaline specific (Hooykaas and Beijersbergen, 1994). Plant genetic transformation by *A. tumefaciens* requires the presence of two genetic components located on the bacterial Ti plasmid: (i) T-DNA, the actual genetic element transferred into the plant cell genome; and (ii) the virulence (*vir*) region composed of seven major loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virG* and *virH*), encoding most components of the protein machinery mediating T-DNA transfer. In addition, a set of *A. tumefaciens* chromosomal virulence (*chv*) genes participates in the early stages of the bacterial chemotaxis and attachment to the plant cells (reviewed by Citovsky *et al*., 1992a; Hooykaas and Beijersbergen, 1994; Sheng and Citovsky, 1996; Zambryski, 1992; Zambryski *et al*., 1989).

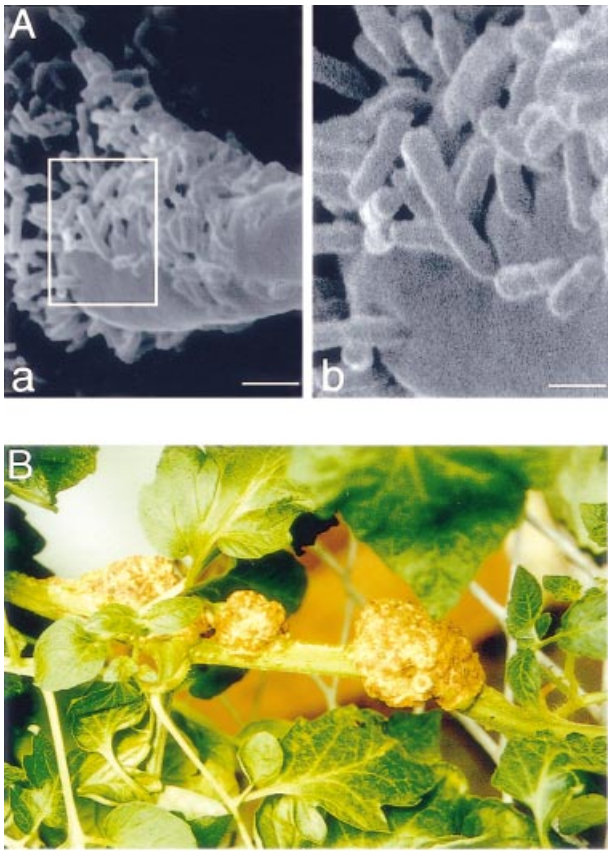


Fig. 1 *Agrobacterium tumefaciens*, bacterium and disease. (A) Scanning electron micrograph of wild-type *A. tumefaciens* cells colonizing aspen roots at two magnifications: (a) bar = 250 μm; (b) bar = 80 μm. (B) Crown gall formation on a stem of a tomato plant infected with wild-type *A. tumefaciens*.

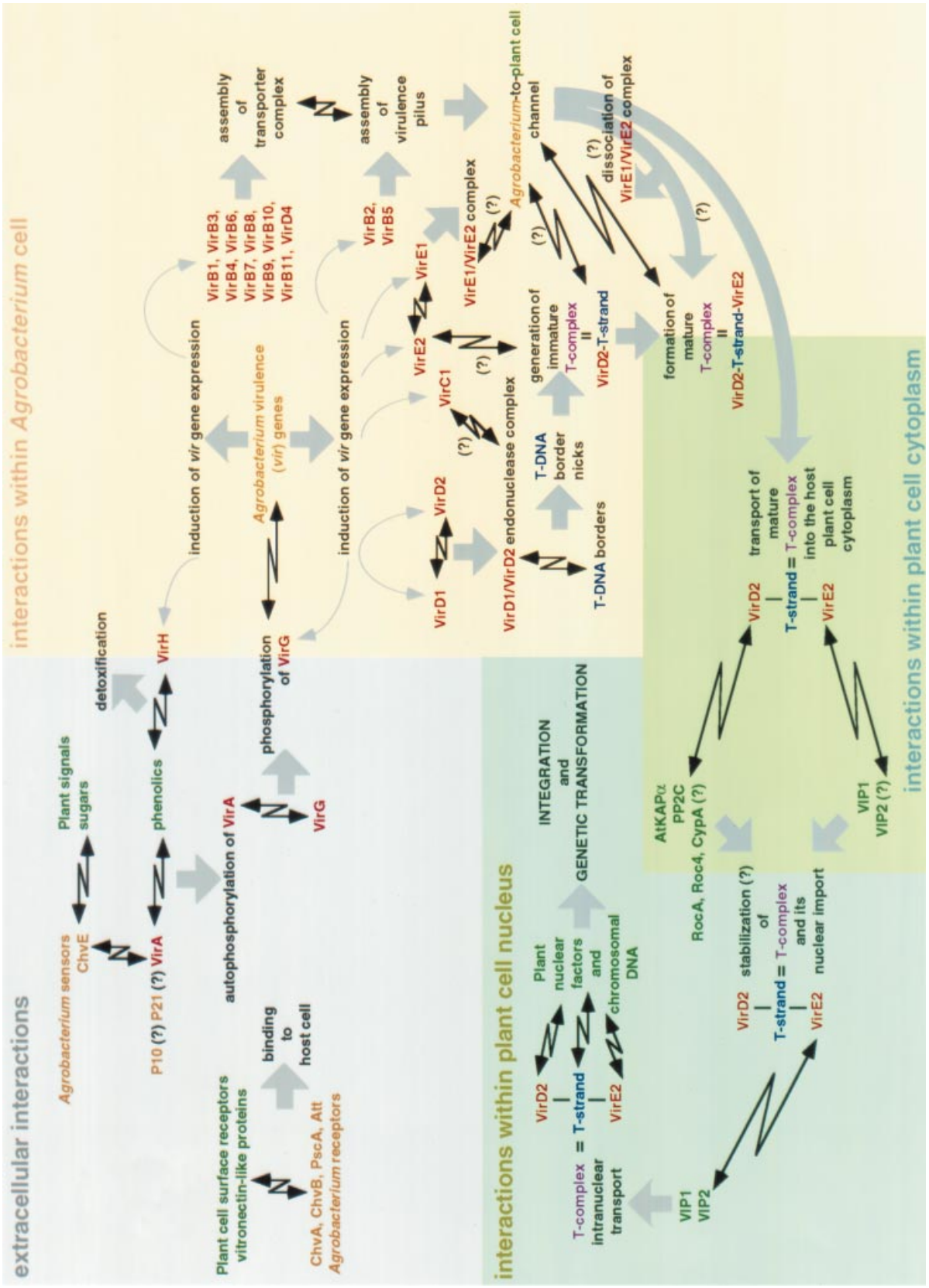
As an organism capable of trans-kingdom DNA transfer (Stachel and Zambryski, 1989), *A. tumefaciens* has been extensively studied. Furthermore, disarmed strains that lack the wild-type T-DNA are widely used in plant genetic engineering (Armitage *et al*., 1988; Bevan, 1984). However, our understanding of the molecular pathways by which T-DNA from *A. tumefaciens* is transferred to plant cells is still incomplete. While the bacterial factors necessary for the infection are relatively well characterized and the complete sequences of the nopaline-type T-DNA region and even the entire Ti plasmid have recently been determined (Gielen *et al*., 1999; Suzuki *et al.,* 2000), much less is known about the host cell factors involved in this process.

Here, we describe the major functional steps of the *A. tumefaciens*–plant cell interaction, emphasizing the role of the bacterial and plant proteins in each step (Fig. 2). Specifically, we focus on the following seven events: (i) *A. tumefaciens*–plant cell recognition and attachment; (ii) sensing of plant signals by *A. tumefaciens*; (iii) activation of *A. tumefaciens vir* genes following transduction of the sensed signals; (iv) generation of a mobile T-DNA; (v) T-DNA export from the bacterial cell; (vi) T-DNA import into the host plant cell nucleus; and (vii) T-DNA integration into the host genome.

A. TUMEFACIENS–PLANT CELL RECOGNITION AND ATTACHMENT

A. tumefaciens recognition of and attachment to the host cells is an early and essential step of the infection process. Following its loose binding to the host, the bacterium synthesizes cellulose filaments which anchor it to the plant cell surface (Matthysse,

Fig. 2 (Opposite) Molecular interactions during T-DNA transfer from *A. tumefaciens* to the plant. Compartments within which different interactions take place are shown by the following backgrounds: grey, extracellular interactions; beige, interactions within the bacterial cell; green, interactions in the host cell cytoplasm; blue–green, interactions in the host cell nucleus. Plant signals and proteins are shown in green, bacterial proteins encoded by chromosomal genes are shown in brown, Vir proteins are shown in red and T-strand and T-complex are shown in blue and purple, respectively. Zigzagged double arrows indicate protein–protein, protein–DNA or protein–signal interactions, while thick grey arrows indicate the functional outcome of these interactions. Thin grey arrows indicate the origin of the indicated Vir proteins. *Summary of molecular interactions*. Bacterial ChvA, ChvB, PscA and Att proteins recognize plant cell surface receptors, resulting in *A. tumefaciens* attachment to the host cell. ChvE reacts with sugar signals and transfers the signals to VirA, which (directly or via bacterial P10 and P21) reacts with phenolic signals. Both signalling pathways result in VirA autophosphorylation. Transfer of the phosphate group from VirA to VirG transmits the plant signals into the bacterial cell, where VirG activates expression of the bacterial *vir* genes. VirH likely functions to detoxify excess signal phenolics. VirB1, VirB3, VirB4, VirB6, VirB7, VirB8, VirB9, VirB10, VirB11 and VirD4 assemble into a transmembrane transporter complex which, together with the VirB2/VirB5 virulence pilus, function as a bacterium-to-plant cell channel. VirD1 complexes with VirD2 into an endonuclease complex that, with the help of VirC1, nicks the antisense strand of the T-DNA at the T-DNA borders. During nicking, VirD2 covalently attaches to the 5′ end of the T-strand, generating an immature T-complex. This complex can mature into the complete T-complex by two different mechanisms. (1) The immature T-complex and VirE2 complexed with VirE1 are separately exported through the same channel. In the host cell cytoplasm, VirE2 dissociates from VirE1 and binds to the T-strand, forming a mature T-complex of VirD2, T-strand and VirE2. (2) VirE2 binds the T-strand already in the bacterial cell, preventing its re-annealing to re-form the Ti plasmid, and the mature T-complex is then exported. VirD2 of the mature T-complex interacts with RocA, Roc4 and CypA cyclophilins to maintain its conformation, with AtKAPα to import it into the host cell nucleus, and with PP2C, perhaps, to regulate import. VirE2 of the mature T-complex interacts with VIP1 to help its nuclear import and, potentially, with VIP2 for its later function in the nucleus (alternatively, VirE2 may interact with VIP2 within the nucleus). VirD2–AtKAPα and VirE2–VIP1 interactions result in efficient nuclear import of the entire T-complex. In the nucleus, VIP1 and/or VIP2 may participate in intranuclear transport of the T-complex, leading it to the integration site in the plant chromosome. Finally, additional nuclear factors act in concert with VirD2 and VirE2 to integrate the T-strand into the chromosomal DNA, resulting in stable genetic transformation of the host plant cell.


extracellular interactions
interactions within Agrobacterium cell
interactions within plant cell nucleus
interactions within plant cell cytoplasm
Agrobacterium sensors
ChvE
Plant signals
sugars
detoxification
P10 (?) P21 (?) VirA
phenolics
VirH
autophosphorylation of VirA
phosphorylation of VirG
VirG
Plant cell surface receptors
vitronectin-like proteins
ChvA, ChvB, PscA, Att
Agrobacterium receptors
binding to host cell
induction of vir gene expression
Agrobacterium virulence (vir) genes
VirB1, VirB3, VirB4, VirB6, VirB7, VirB8, VirB9, VirB10, VirB11, VirD4
assembly of transporter complex
VirB2, VirB5
assembly of virulence pilus
VirE2
VirE1
VirE1/VirE2 complex
Agrobacterium-to-plant cell channel
VirC1
VirD1
VirD2
VirD1/VirD2 endonuclease complex
T-DNA border nicks
T-DNA borders
generation of immature T-complex
VirD2-T-strand
formation of mature T-complex
VirD2-T-strand-VirE2
dissociation of VirE1/VirE2 complex
INTEGRATION and GENETIC TRANSFORMATION
T-complex = T-strand
intranuclear transport
VirD2
VirE2
Plant nuclear factors and chromosomal DNA
VIP1
VIP2
RocA, Roc4, CypA (?)
AtKAPα
PP2C
stabilization (?) of T-strand = T-complex and its nuclear import
transport of mature T-strand = T-complex into the host plant cell cytoplasm
VIP1
VIP2 (?)

1986). The isolation of *A. tumefaciens* mutants unable to bind to plant cells identified several chromosomal genes, e.g. *chvA*, *chvB*, *pscA*, *att* (Fig. 2, extracellular interactions), required for this attachment process. Conversely, plant molecules recognized by *A. tumefaciens* and involved in its attachment are still poorly characterized. One such surface receptor may belong to a vitronectin protein family. In animal cells, vitronectin, a component of the extracellular matrix, is utilized as a specific receptor by several pathogenic bacterial strains (Paulsson and Wadstrom, 1990). Similarly, plant vitronectin-like molecules might be involved in the *A. tumefaciens*–plant cell binding. Indeed, *A. tumefaciens* attachment to plant cells was blocked by human vitronectin or antivitronectin antibodies. In addition, *A. tumefaciens chvB*, *pscA* and *att* mutants unable to bind plant cells exhibited reduced binding to vitronectin (Wagner and Matthysse, 1992). Interestingly, while plant and animal vitronectins are related immunologically, they do not share an amino acid sequence similarity (Zhu *et al*., 1994).

In addition to plant vitronectins, other cell surface molecules may be recognized by *A. tumefaciens*. Recent identification of several T-DNA-tagged *Arabidopsis thaliana* mutants defective in their ability to bind *A. tumefaciens* (Nam *et al*., 1999) will most likely lead to the comprehensive identification and study of plant proteins and carbohydrates involved in the binding of *A. tumefaciens*.

SENSING PLANT SIGNALS

A. tumefaciens has adopted a two-component regulatory system, typical of many prokaryotes (reviewed by Stock *et al*., 1990), to sense and react to the presence of the susceptible host cells. The system components, a membrane sensor protein, VirA, and a cytoplasmic response regulator protein, VirG (Fig. 2, extracellular interactions), specifically react to the presence of wounded plant cell exudates and promote transcriptional activation of the *vir* genes (Winans *et al*., 1994). Among various phenolic compounds, e.g. lignin and flavonoid precursors, known to induce *vir* gene expression, the best-studied inducer is acetosyringone, a monocyclic phenolic molecule (Stachel *et al*., 1985). Monosaccharides, such as glucose and galactose, have also been shown to induce *vir* gene expression, when acetosyringone was absent or present in limited quantities (Cangelosi *et al*., 1990).

To initiate the signalling pathway, plant phenolics interact with the transmembrane sensor protein, VirA. This interaction may be either direct or indirect, mediated by two chromosomally encoded proteins, P10 and P21 (Lee *et al*., 1992), which first bind the phenolic signal and then transmit it to VirA. That phenolic signals are recognized directly by VirA was first supported by the observation that all *A. tumefaciens* mutants unable to respond to the phenolic signals are defective in *virA* (Lee *et al.,* 1995). Furthermore, when different Ti plasmids were transferred into isogenic chromosomal backgrounds, the phenolic sensing determinants were associated with the Ti plasmid, and not with the chromosomal background (Lee *et al*., 1996), providing additional evidence for direct recognition of phenolic signals by VirA. In contrast, the induction of *vir* genes by sugars always occurs indirectly, i.e. through a chromosomally encoded glucose/galactose binding protein, ChvE, which interacts with VirA and transmits the signal (Shimoda *et al*., 1993). ChvE was also found to broaden the phenolic recognition profiles of the VirA protein, when expressed at high levels (Peng *et al*., 1998), allowing agroinfection of maize (Heath *et al*., 1997).

Mutational analysis of VirA revealed that most of its periplasmic domain is responsible for the interaction with and response to ChvE (Doty *et al*., 1996). Mutations in the transmembrane domains and the amphiphathic helix of VirA resulted in defective response to acetosyringone, but did not affect protein stability, topology or ability to dimerize. Thus, these mutations were suggested to interfere specifically with the detection of phenolic inducers or transmission of the signals to the VirA kinase domain (Doty *et al*., 1996). However, since the acetosyringone receptor domain had previously been mapped to the cytoplasmic part of VirA (Turk *et al*., 1994), the transmembrane domains and the amphiphathic helix of VirA are most likely involved in signal transmission.

Like many other bacterial sensor proteins, VirA is autophosphorylated (Jin *et al*., 1990b). This phosphorylation occurs at a specific histidine residue within the C-terminal half of the protein which also contains the VirA protein kinase domain (reviewed by Sheng and Citovsky, 1996; Winans *et al*., 1994). Because the high-energy phosphate bond of phosphohistidine is unstable (Pollard and Cooper, 1986), it is then transferred to an aspartate residue(s) of VirG, a cytoplasmic transducer protein, which shares amino acid similarity with many other transducer proteins, yet is uniquely stable in its phosphorylated form (Jin *et al*., 1990a), probably to allow maximal levels of *vir* gene induction. Thus, during signal transduction, VirA functions both as a protein kinase and as a phosphotransferase.

Plant phenolics are bacteriostatic at high concentrations, representing a part of the plant defence systems against pathogens. To avoid this defensive mechanism, the protein products of the *virH* (or *pinF*) locus, which share sequence similarity with cytochrome P450 (Kanemoto *et al*., 1989), likely function to detoxify these harmful phenolics; for example, a phenolic *vir* gene inducer, ferulic acid, is detoxified by the Virh2 protein (Kalogeraki *et al*., 1999). Since *virH* expression is induced by VirG (Fig. 2, interactions within the *Agrobacterium* cell) which, in turn, is activated in the presence of the phenolics, the detoxification will occur only after these plant molecules have fulfilled their signalling role in *A. tumefaciens* infection.

INDUCTION OF *VIR* GENES

The phosphorylated VirG protein specifically interacts with the *vir* box, a conserved 12-bp sequence located in the promoter

regions of the *vir* genes (Fig. 2, interactions within the *Agrobacterium* cell); because the *vir* box is partly symmetrical, VirG may bind it as a dimer. Although unphosphorylated VirG still binds to *vir* boxes, the phosphorylation step is required for the activation of *vir* gene expression, potentially by enhancing the ability of VirG to recruit other proteins involved in transcriptional activation. It is also possible that phosphorylated VirG has a higher DNA binding affinity (Jin *et al*., 1990c), as reported for other promoter binding proteins (Kato *et al*., 1989; Ninfa and Magasanik, 1986).

In addition to that of other *vir* genes, VirG activates its own expression (Stachel and Zambryski, 1986). Constitutively expressed VirG is translated from mRNA produced both in the absence and in the presence of plant signals. This protein is likely required to initially transduce the signal from VirA. Following induction by plant phenolics, a message longer by 50 nts is produced at 10-fold higher levels than the constitutive shorter transcript (Zambryski, 1989), generating more VirG and allowing efficient activation of other *vir* genes.

GENERATION OF A MOBILE T-DNA COPY, THE T-STRAND

Expression of *vir* genes leads to the production of a single-stranded T-DNA copy, termed the T-strand (Stachel *et al*., 1986), which is then transported into the host cell. Different Ti plasmids carry different numbers and sizes of T-DNA elements. For example, nopaline Ti plasmids contain a single ≈ 22-kb T-DNA, while octopine Ti plasmids carry three shorter T-DNAs of 13, 1.5 and 7.8 kb which are transported independently of each other. Regardless of their size and origin, all T-DNA elements are delimited by conserved 25-bp sequences that define their right and left borders (Zambryski *et al*., 1982). Genetic studies showed that while the right border is absolutely required for *A. tumefaciens* pathogenicity, the left border is not (Shaw *et al*., 1984; Wang *et al*., 1984).

T-strand production initiates at the T-DNA right border, continues in the 5′ to 3′ direction, terminating at the left border. A complex of VirD2 and VirD1 proteins (Fig. 2, interactions within the *Agrobacterium* cell) functions as a site-and strand-specific endonuclease which binds the supercoiled Ti plasmid at the T-DNA borders, relaxes it and nicks the antisense T-DNA strand between the third and fourth bases of the T-DNA borders (Filichkin and Gelvin, 1993; Scheiffele *et al*., 1995; Wang *et al*., 1987). When VirD2 and VirD1 are limiting, T-strand production can be enhanced by another virulence protein, VirC1 (De Vos and Zambryski, 1989). Following the nicking reaction, VirD2 becomes covalently attached to the 5′ end of the T-strand and to the exposed 5′ end of the antisense strand at the left border nick (Herrera-Estrella *et al*., 1988; Howard *et al*., 1989; Ward and Barnes, 1988; Young and Nester, 1988). The resulting T-strand is then removed while the bacterial DNA synthesis machinery repairs the gap between the right and left borders of the antisense strand (Pansegrau *et al*., 1993); it is still unclear whether the release of the T-strand and the gap repair occur simultaneously or consecutively. The VirD2 molecule attached to the left border nick on the Ti plasmid may then ligate this nick, restoring the intact Ti plasmid. The VirD2 molecule attached to the T-strand remains with it throughout its voyage into the plant cell, providing it with transport signals and protection (see below). VirD1, on the other hand, does not remain bound to the T-strand, and its role is probably limited to T-DNA border nicking.

To facilitate its movement, the T-strand molecule must be prevented from re-annealing to its sense strand. Thus, the nascent T-strand should either be transported into the host plant cell concomitantly with its unwinding or, by analogy with prokaryotic DNA replication systems (Chase and Williams, 1986), protected by a single-stranded (ss) DNA binding protein (SSB). That T-strands accumulate within vir-induced A. tumefaciens, even in the absence of the recipient plant cells (Stachel *et al*., 1986, 1987), suggests involvement of SSB. Indeed, VirE2 has been shown to bind ssDNA (Christie *et al*., 1988; Citovsky *et al*., 1988, 1989; Das, 1988; Gietl *et al*., 1987). VirE2 packages ssDNA into semirigid, hollow, cylindrical filaments with a telephone cord-like structure. Mass analysis of these filaments predicted that one 22-kb nopaline-specific T-strand would contain 1176 molecules of VirE2 and have an outer diameter of 12.6 nm (Citovsky *et al*., 1997). Thus, VirE2 has been proposed to coat the newly made T-strand and form, together with the T-strand-associated VirD2 molecule, the T-DNA transfer (T)-complex (Howard and Citovsky, 1990; Zupan and Zambryski, 1997).

Cooperative interaction between VirE2 and ssDNA (Citovsky *et al*., 1989) and their likely proximity during VirE2 synthesis and T-strand production support the idea that VirE2 binds to the T-strand early in the infection (Fig. 2, interactions within the *Agrobacterium* cell), especially if both of them are then exported into the host cell through the same channel (as suggested by Binns *et al*., 1995). Indeed, in *A. tumefaciens* extracts, T-strands and VirE2 are coprecipitated by anti-VirE2 antibodies (Christie *et al*., 1988). Yet, the hypothesis that the T-strand travels as a complete T-complex from the bacterium to the host cell (Fig. 2, interactions within the plant cell cytoplasm) was challenged by the finding that the coinoculation of plants with one *A. tumefaciens* strain that contained T-DNA, but lacked VirE2, and another strain that lacked T-DNA, but contained VirE2, restored infectivity to these individually nonpathogenic bacteria, suggesting that T-strands and VirE2 are exported independently of each other (Otten *et al*., 1984). Similarly, VirE2 supplied from transgenic plants restored the tumorigenicity of a VirE2 mutant *A. tumefaciens* strain (Citovsky *et al*., 1992b). Consistently, VirE2 export into plants can be inhibited without affecting the export of the T-DNA (Binns *et al*., 1995; Lee *et al*., 1999). In addition, recent studies suggested

that VirE1, another product of the *virE* locus, binds to VirE2, prevents VirE2 binding to ssDNA and facilitates VirE2 but not T-strand export into plant cells (Deng *et al*., 1999; Sundberg and Ream, 1999; Sundberg *et al*., 1996; Zhou and Christie, 1999). Thus, VirE1 may export VirE2 into the host cell cytoplasm where these two proteins could dissociate from each other, allowing VirE2 to bind to the T-strands. In this model, VirE2 and an immature T-complex, i.e. T-strand associated with VirD2, independently travel into the host plant cell, within which the mature T-complex is then assembled.

T-COMPLEX EXPORT MACHINERY

Whether the T-strand travels as a mature or immature T-complex, it requires a specific export system to deliver it across the bacterial envelope and the plasma membrane of the host plant cell. Recent data indicate that the T-complex transport occurs by a type IV secretion system (reviewed by Christie, 1997; Zupan *et al*., 1998). Type IV systems (originally defined by Salmond, 1994) usually contain up to 12 proteins which form two functional components: a filamentous pilus and a transporter complex that translocates substrates through the cell membranes. In *A. tumefaciens*, the type IV transport apparatus is assembled from proteins encoded by the *virD4* gene and *virB* operon, with 11 open reading frames (reviewed by Christie, 1997; Zupan *et al*., 1998).

The pilus component of the T-complex transport system has been observed on the surface of *vir*-induced *A. tumefaciens* cells (Fullner *et al*., 1996). Structurally, this virulence pilus most likely is composed of two proteins, VirB2 (Lai and Kado, 1998) and VirB5 (Shirasu and Kado, 1993). By analogy to the F-pilus, the best-studied component of type IV systems that mediates cell surface contact during bacterial conjugation (Firth *et al*., 1996), VirB2 represents a major and VirB5 a minor protein constituent of the *A. tumefaciens* pilus (Zupan *et al*., 1998). Functionally, the VirB pilus was proposed to sense contact with the recipient plant cell and transduce this information to the transporter complex to initiate export of the T-complex (Zupan *et al*., 1998).

The transporter complex is composed of various VirB proteins and VirD4 (Fig. 2, interactions within the *Agrobacterium* cell). In this structure, VirB1 is thought to utilize its transglycolsylase activity to locally dissolve the bacterial peptidoglycan layer in preparation of the site for the assembly of the transporter complex within the bacterial envelope (Baron *et al*., 1997; Mushegian *et al*., 1996). In addition, a smaller, processed form of VirB1, designated VirB1* (Baron *et al*., 1997), is secreted to the cell exterior by an as yet unknown mechanism which involves an N-terminal signal peptide of VirB1 (Zupan *et al*., 1998). The secreted VirB1* may then participate in establishing cell–cell contact with the recipient plant cell (Christie, 1997).

Based on their homology to the F-pilus formation machinery, VirB3 and VirB4 components of the transporter have been proposed to promote assembly of the virulence pilus (Zupan *et al*., 1998). While VirB3 is able to cross bacterial membranes, VirB4 is a transmembrane protein with an ATPase activity (reviewed by Christie, 1997). In addition, VirB4 with another ATPase, VirB11 (Christie *et al*., 1989; Shirasu *et al*., 1994), may activate the transporter by coupling the energy of ATP hydrolysis to T-complex transport (Christie, 1997).

Another transmembrane component of the transporter complex is VirB6 (Fig. 2, interactions within the *Agrobacterium* cell). Although its exact function during transport is unknown, the presence of six transmembrane domains (Zambryski, 1992) suggests a role in the formation of the transporter pore (Christie, 1997). VirB7 and VirB9 have been shown to covalently dimerize into VirB7/VirB7 homodimers and VirB7/VirB9 heterodimers (Baron *et al*., 1997; Das *et al*., 1997; Spudich *et al*., 1996), becoming a nucleation centre for the rest of the transporter complex (Christie, 1997; Zupan *et al*., 1998). Both proteins are located in the outer membrane of the bacterial envelope, probably anchored by the lipid moiety of VirB7 (Baron *et al*., 1997; Fernandez *et al*., 1996).

While the function of VirB8 remains unknown, it has been found on the periplasmic face of the inner bacterial membrane (reviewed by Christie, 1997), suggesting its involvement with the transport apparatus. Similarly, the function of the transmembrane VirB10 protein is unknown. Genetic evidence suggests interactions between VirB10 and the VirB7/VirB9 nucleation centre of the transporter (Beaupre *et al*., 1997); this interaction, however, has not been verified biochemically (Zupan *et al*., 1998).

The final component of the transporter complex is VirD4 (Fig. 2, interactions within the *Agrobacterium* cell), which is required for formation of the virulence pilus (Fullner *et al*., 1996). In addition, VirD4, a transmembrane ATPase (reviewed by Christie, 1997), is thought to couple T-strand formation to its transport by introducing the newly made T-complex into the transporter using an energy-dependent mechanism (Christie, 1997; Zupan *et al*., 1998).

Assembly of the virulence pilus and the transporter complex may not suffice to initiate export of the T-complexes. A recent model for the T-complex transport suggests that it is activated by physical contact with the recipient plant cell (Zupan *et al*., 1998). Specifically, in the absence of such contact, the channel is closed, resulting in the accumulation of the T-complexes within the bacterial cell. When the virulence pilus contacts a putative receptor on the plant cell surface, the channel opens, probably via a change in conformation, allowing transport to occur (Zupan *et al*., 1998). Following this event, the T-complex is exported from the bacterial cell into the host plant cell cytoplasm. As mentioned above, the same transporter channel may also serve to export free VirE2 molecules (Binns *et al*., 1995) or VirE2/VirE1 heterodimers.

NUCLEAR IMPORT OF THE T-COMPLEX

The last barrier in the T-complex journey to the plant chromosome is the plant nuclear envelope. The large size of the T-complex (12.6 nm in diameter, see Citovsky *et al.*, 1997) exceeds the diameter of diffusion channels of the nuclear pore (9 nm, reviewed by Forbes, 1992). However, the size exclusion limit of the nuclear pore can open to 23 nm during the process of active nuclear uptake (Dworetzky and Feldherr, 1988; Forbes, 1992). Thus, the T-complex is most likely actively imported into the host cell nucleus.

Unlike other mobile genetic elements (e.g. retroviruses and transposons), T-DNA does not encode protein functions required for its transport and integration. Thus, T-DNA is sequence non-specific; any DNA sequence inserted between the T-DNA borders will be transferred to the host plant cell and integrated into its genome (reviewed by Citovsky *et al.*, 1992a; Zambryski, 1992). This lack of specificity allows the use of infection by *A. tumefaciens* harbouring a disarmed Ti plasmid, which lacks T-DNA but contains active *vir* genes, and a binary plasmid, carrying the gene of interest between the T-DNA borders, for the efficient production of transgenic plants (Armitage *et al.*, 1988).

Increasing evidence suggests that both protein components of the T-complex, i.e. VirD2 and VirE2, directly mediate the process of its nuclear import. Numerous studies have shown that VirD2 specifically accumulates in the plant cell nucleus (Citovsky *et al.*, 1994; Herrera-Estrella *et al.*, 1990; Howard *et al.*, 1992; Rossi *et al.*, 1993; Tinland *et al.*, 1992). Although VirD2 appears to contain two nuclear localization signals (NLS), one at each end of the molecule (Herrera-Estrella *et al.*, 1990; Howard *et al.*, 1992), only the C-terminal sequence, belonging to a bipartite class of NLS (Dingwall and Laskey, 1991; Howard *et al.*, 1992), functions during *A. tumefaciens* infection (Koukolikova-Nicola *et al.*, 1993; Mysore *et al.*, 1998; Rossi *et al.*, 1993; Shurvinton *et al.*, 1992). The VirD2 role in T-complex nuclear import was confirmed by studies showing that T-DNA expression and tumorigenicity are reduced in *A. tumefaciens* strains carrying NLS-deletion mutants of VirD2 (Narasimhulu *et al.*, 1996; Shurvinton *et al.*, 1992).

The observation that the deletion of VirD2 NLS reduces rather than abolishes tumorigenicity suggests that another T-complex component, such as VirE2, helps nuclear import. Indeed, VirE2 accumulated in the nuclei of plant cells and this nuclear import was mediated by protein sequences within the middle part of the VirE2 molecule (Citovsky *et al.*, 1992b, 1994). Consistently, mutations in the central region of VirE2 decreased tumorigenicity but did not affect ssDNA binding or stability of this protein (Dombek and Ream, 1997). The involvement of VirE2 in T-DNA nuclear import was then demonstrated by microinjection of an *in vitro*-formed complex of VirE2 and fluorescently labelled ssDNA, which promoted the efficient nuclear accumulation of the labelled ssDNA (Zupan *et al.*, 1996). Microinjection of fluorescent ssDNA alone resulted in only cytoplasmic but no nuclear fluorescence; furthermore, VirE2 did not import fluorescently labelled double-stranded DNA, indicating a need for the formation of nucleoprotein complexes. Finally, nuclear import of VirE2 ssDNA was blocked by known specific inhibitors of nuclear import (Zupan *et al.*, 1996). Second, an *A. tumefaciens* strain, lacking the entire VirE2 as well as the specific C-terminal NLS of VirD2, was not infectious on wild-type tobacco, but produced tumours on VirE2-expressing transgenic plants (Gelvin, 1998). Thus, VirE2 expressed in a plant cell transferred T-strands into the nucleus in the absence of an NLS from any other known T-DNA-associated protein (Gelvin, 1998).

Interestingly, whereas both octopine- and nopaline-specific VirD2 are imported by a mechanism conserved between animal, yeast and plant cells (Citovsky *et al.*, 1994; Guralnick *et al.*, 1996; Howard *et al.*, 1992; Rhee *et al.,* 2000; Ziemienowicz *et al.*, 1999), nuclear localization of the nopaline-type VirE2 was plant specific, and did not occur in animal or yeast cells (Citovsky *et al.*, 1992b, 1994; Guralnick *et al.*, 1996; Rhee *et al.,* 2000). On the other hand, octopine VirE2 has been shown to enter animal cell nuclei *in vitro* (Ziemienowicz *et al.*, 1999), suggesting that nopaline and octopine VirE2 proteins may differ in their ability to function in nonplant systems. Alternatively, nuclear import of VirE2 in a cell-free system may differ from that within living cells. Interestingly, however, octopine VirE2 function in animal cells may still be impaired, as it alone was unable to mediate nuclear import of ssDNA in this system (Ziemienowicz *et al.*, 1999).

In the plant cell, VirD2 and VirE2 must interact with host factors potentially involved in the T-complex nuclear import and integration (Fig. 2, interactions within the *Agrobacterium* cell). VirD2 was found to interact with three isoforms of *A. thaliana* cyclophilins, RocA, Roc4 and CypA (Deng *et al.*, 1998). *In vitro* inhibition of VirD2–CypA interaction by cyclosporin abolished *A. tumefaciens* tumorigenicity in *A. thaliana* and tobacco. Because cyclophilins are known to act as protein chaperones, RocA, Roc4 and CypA were proposed to maintain the proper conformation of VirD2 in the host cell cytoplasm and/or nucleus (Fig. 2, interactions within the plant cell cytoplasm, interactions within the plant nucleus) during the T-DNA nuclear import and/or integration (Deng *et al.*, 1998).

Another VirD2-interacting plant protein is a type 2C serine/threonine protein phosphatase (PP2C) (Y. Tao, P. Rao and S. Gelvin, unpublished results). An *A. thaliana* PP2C mutant *abi1* (Meyer *et al.,* 1994) has an increased susceptibility to *A. tumefaciens*, whereas overexpression of PP2C in tobacco protoplasts inhibits nuclear accumulation of GUS–VirD2 NLS fusion, suggesting a role for PP2C in VirD2 nuclear import (Y. Tao, P. Rao and S. Gelvin, unpublished results).

Finally, VirD2 was found to interact with AtKAPα (Ballas and Citovsky, 1997), a member of the *A. thaliana* karyopherin α family (Fig. 2, interactions within the plant cell cytoplasm), proteins known to mediate the nuclear import of NLS-containing proteins

(reviewed in Nakielny and Dreyfuss, 1999). The role of AtKAPα in VirD2 nuclear import was demonstrated directly when AtKAPα promoted the nuclear accumulation of fluorescently labelled VirD2 in permeabilized yeast cells (Ballas and Citovsky, 1997). Thus, AtKAPα may help lead the VirD2 and its cognate T-strand into the cell nucleus.

Unlike VirD2, VirE2 did not interact with AtKAPα (Ballas and Citovsky, 1997), but specifically interacted with two *A. thaliana* proteins, VIP1 and VIP2 (T. Tzfira and V. Citovsky, unpublished results; Fig. 2, interactions within the plant cell cytoplasm). Using a genetic assay for nuclear import (Rhee *et al.,* 2000), expression of VIP1 was shown to allow the nuclear import of nopaline-type VirE2 in yeast, and coexpression of VIP1 promoted nuclear import of GFP–VirE2 in animal cells (T. Tzfira and V. Citovsky, unpublished results). Interestingly, VIP1, a bZIP protein, had no homology to known animal or yeast proteins, suggesting that VIP1 may represent a cellular factor involved in the plant-specific nuclear import of nopaline VirE2.

The role of VIP1 in the T-complex nuclear import was further studied in transgenic tobacco plants expressing VIP1 in the antisense orientation. These plants exhibited dramatically reduced tumorigenicity when infected with wild-type *A. tumefaciens*. Furthermore, the VIP1 antisense plants displayed low levels of transient expression of the GUS reporter gene when infected with an *A. tumefaciens* strain carrying an intron-containing GUS gene. No reduction in GUS gene expression, however, was observed when the reporter plasmid was delivered biolistically (T. Tzfira and V. Citovsky, unpublished results). These results suggest that VIP1 is involved in the early stages of T-DNA expression, potentially in its nuclear import.

The C-terminus of VIP2 is similar to the *Drosophila* Rga protein (T. Tzfira and V. Citovsky, unpublished results), which is thought to mediate interaction between chromatin proteins and transcriptional complexes (Frolov *et al.*, 1998). Potentially, VIP2 may recognize and associate with the plant chromatin. Although VIP2 had no effect on the intracellular localization of VirE2 in yeast or mammalian cells, it interacted not only with VirE2 but also with VIP1 in the two-hybrid system (T. Tzfira and V. Citovsky, unpublished results). Thus, VIP1, VIP2 and VirE2 may function in a multiprotein complex that performs a dual role: facilitating nuclear targeting of VirE2 and participating in intranuclear transport of VirE2 and possibly the T-complex to the site of integration. A similar dual role in nuclear and intranuclear transport has been suggested for the yeast Kap114p protein that imports the TATA binding protein (TBP) into the cell nucleus and targets it to the promoters of genes to be transcribed (Pemberton *et al.*, 1999).

T-DNA INTEGRATION

Once in the nucleus, the T-complex integrates into the plant chromosome. As the T-DNA does not encode the enzymatic activities required for integration, VirD2 and VirE2 proteins and/or host nuclear factors must provide these activities. Indeed, VirD2, which has a ligase activity *in vitro* (Pansegrau *et al.*, 1993), was proposed to participate in the ligation of the 5′ end of the T-DNA to the genomic DNA, followed by second strand synthesis by the plant DNA repair machinery (Tinland *et al.*, 1995). However, another study suggested that the T-strand is converted into a double-stranded form prior to integration (De Neve *et al.*, 1997). Besides its ligase activity, VirD2 contains two amino acid sequence motifs potentially involved in the integration process: the C-terminal ω domain (Shurvinton *et al.*, 1992) and a motif common to the integrase family of recombinases (Argos *et al.*, 1986). Deletion of the ω domain significantly reduces integration (Mysore *et al.*, 1998; Narasimhulu *et al.*, 1996), whereas mutations (e.g. R129G substitution) in the integrase motif were found to decrease the precision but not the efficiency of integration (Tinland *et al.*, 1995). In addition to VirD2, VirE2 may also be involved in the integration process. Specifically, VirE2 may be required for integration fidelity of the 3′ end of the T-strand (Rossi *et al.*, 1996).

The search for plant factors involved in the T-DNA integration (Fig. 2, interactions within the plant cell nucleus) led to the identification and isolation of several *Arabidopsis* mutants, which are resistant to *A. tumefaciens* transformation at different stages of the transformation process (Mysore *et al.,* 2000a,b; Nam *et al.*, 1999). Mutant *rat5*, defective for stable but not transient T-DNA expression, maps to the H2A histone gene, suggesting a role of the H2A histone in T-DNA integration (Mysore *et al.,* 2000b). Because T-DNA integration deficiency has been suggested to underlie the resistance of several *A. thaliana* ecotypes to *A. tumefaciens* infection (Nam *et al.*, 1997), other plant factors are most likely involved in the T-DNA integration process.

FUTURE OUTLOOK

The significant progress achieved in our understanding of the molecular interactions between *A. tumefaciens* and plant cells has mainly been derived from studies of the bacterial virulence proteins. Identification of the host cellular factors involved in these processes, although revealing, is just beginning. Thus, future developments in this field will most likely come from the isolation of additional cellular participants and regulatory components of the infection and genetic transformation pathways. For instance, cellular proteins that interact with VirD2 and/or VirE2 during T-DNA integration remain unknown. Perhaps the best way to achieve these goals is to combine biochemical, molecular and cell biological techniques with a genetic approach aimed at identifying and characterizing plant mutants with altered susceptibility to *A. tumefaciens* infection. The resulting knowledge will help the design of new strategies to produce agronomically

important plants resistant to *A. tumefaciens*, and allow the development of improved genetic engineering techniques for efficient nuclear delivery and integration of foreign genes.

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REFERENCES

Argos, P., Landy, A., Abremski, K., Egan, J.B., Haggard-Ljungquist, E., Hoess, R.H., Kahn, M.L., Kalionis, B., Narayana, S.V., Pierson, L.S.D., Sternberg, N. and Leong, J.M. (1986) The integrase family of site-specific recombinases: regional similarities and global diversity. *EMBO J.* **5**, 433–440.

Armitage, P., Walden, R. and Draper, J. (1988) Vectors for the transformation of plant cells using *Agrobacterium*. In *Plant Genetic Transformation and Gene Expression, A Laboratory Manual* (Draper, J., Scott, R., Armitage, P. and Walden, R., eds). London: Blackwell Scientific Publications, pp. 1–67.

Ballas, N. and Citovsky, V. (1997) Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein. *Proc. Natl Acad Sci. USA*, **94**, 10723–10728.

Baron, C., Llosa, M., Zhou, S. and Zambryski, P.C. (1997) VirB1, a component of the T-complex transfer machinery of *Agrobacterium tumefaciens*, is processed to a C-terminal secreted product, VirB1. *J. Bacteriol.* **179**, 1203–1210.

Beaupre, C.E., Bohne, J., Dale, E.M. and Binns, A.N. (1997) Interactions between VirB9 and VirB10 membrane proteins involved in movement of DNA from *Agrobacterium tumefaciens* into plant cells. *J. Bacteriol.* **179**, 78–89.

Bevan, M.W. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acids Res.* **12**, 1811–1821.

Binns, A.N., Beaupre, C.E. and Dale, E.M. (1995) Inhibition of VirB-mediated transfer of diverse substrates from *Agrobacterium tumefaciens* by the InQ plasmid RSF1010. *J. Bacteriol.* **177**, 4890–4899.

Cangelosi, G.A., Ankenbauer, R.G. and Nester, E.W. (1990) Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. *Proc. Natl Acad. Sci. USA*, **87**, 6708–6712.

Chase, J.W. and Williams, K.R. (1986) Single-stranded DNA binding proteins required for DNA replication. *Annu. Rev. Biochem.* **55**, 103–136.

Christie, P.J. (1997) *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. *J. Bacteriol.* **179**, 3085–3094.

Christie, P.J., Ward, J.E., Gordon, M.P. and Nester, E.W. (1989) A gene required for transfer of T-DNA to plants encodes an ATPase with autophosphorylating activity. *Proc. Natl Acad. Sci. USA*, **86**, 9677–9681.

Christie, P.J., Ward, J.E., Winans, S.C. and Nester, E.W. (1988) The *Agrobacterium tumefaciens virE2* gene product is a single-stranded-DNA-binding protein that associates with T-DNA. *J. Bacteriol.* **170**, 2659–2667.

Citovsky, V., De Vos, G. and Zambryski, P. (1988) Single-stranded DNA binding protein encoded by the *virE* locus of *Agrobacterium tumefaciens*. *Science*, **240**, 501–504.

Citovsky, V., Guralnick, B., Simon, M.N. and Wall, J.S. (1997) The molecular structure of *Agrobacterium* VirE2-single stranded DNA complexes involved in nuclear import. *J. Mol. Biol.* **272**, 718–727.

Citovsky, V., McLean, B.G., Greene, E., Howard, E., Kuldau, G., Thorstenson, Y., Zupan, J. and Zambryski, P. (1992a) *Agrobacterium*–plant cell interaction: induction of *vir* genes and T-DNA transfer. In *Molecular Signals in Plant-Microbe Communications* (Verma, D.P.S., ed.), pp. 169–198. Boca Raton, FL: CRC Press.

Citovsky, V., Warnick, D. and Zambryski, P. (1994) Nuclear import of *Agrobacterium* VirD2 and VirE2 proteins in maize and tobacco. *Proc. Natl Acad. Sci. USA*, **91**, 3210–3214.

Citovsky, V., Wong, M.L. and Zambryski, P. (1989) Cooperative interaction of *Agrobacterium* VirE2 protein with single stranded DNA: implications for the T-DNA transfer process. *Proc. Natl Acad. Sci. USA*, **86**, 1193–1197.

Citovsky, V., Zupan, J., Warnick, D. and Zambryski, P. (1992b) Nuclear localization of *Agrobacterium* VirE2 protein in plant cells. *Science*, **256**, 1803–1805.

Das, A. (1988) *Agrobacterium tumefaciens virE* operon encodes a single-stranded DNA-binding protein. *Proc. Natl Acad. Sci. USA*, **85**, 2909–2913.

Das, A., Anderson, L.B. and Xie, Y.H. (1997) Delineation of the interaction domains of *Agrobacterium tumefaciens* VirB7 and VirB9 by use of the yeast two-hybrid assay. *J. Bacteriol.* **179**, 3404–3409.

de Cleene, M. and de Ley, J. (1976) The host range of crown gall. *Bot. Rev.* **42**, 389–466.

De Neve, M., De Buck, S., Jacobs, A., Van Montagu, M. and Depicker, A. (1997) T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. *Plant J.* **11**, 15–29.

De Vos, G. and Zambryski, P. (1989) Expression of *Agrobacterium* nopaline specific VirD1, VirD2, and VirC1 proteins and their requirement for T-strand production in *E. coli*. *Mol. Plant-Microbe Interact.* **2**, 43–52.

Deng, W., Chen, L., Peng, W.T., Liang, X., Sekiguchi, S., Gordon, M.P., Comai, L. and Nester, E.W. (1999) VirE1 is a specific molecular chaperone for the exported single-stranded-DNA-binding protein VirE2 in *Agrobacterium*. *Mol. Microbiol.* **31**, 1795–1807.

Deng, W., Chen, L., Wood, D.W., Metcalfe, T., Liang, X., Gordon, M.P., Comai, L. and Nester, E.W. (1998) *Agrobacterium* VirD2 protein interacts with plant host cyclophilins. *Proc. Natl Acad. Sci. USA*, **95**, 7040–7045.

Dingwall, C. and Laskey, R.A. (1991) Nuclear targeting sequences—a consensus? *Trends Biochem. Sci.* **16**, 478–481.

Dombek, P. and Ream, W. (1997) Functional domains of *Agrobacterium tumefaciens* single-stranded DNA-binding protein VirE2. *J. Bacteriol.* **179**, 1165–1173.

Doty, S.L., Yu, M.C., Lundin, J.I., Heath, J.D. and Nester, E.W. (1996) Mutational analysis of the input domain of the VirA protein of *Agrobacterium tumefaciens*. *J. Bacteriol.* **178**, 961–970.

Dworetzky, S.I. and Feldherr, C.M. (1988) Translocation of RNA-coated gold particles through the nuclear pores of oocytes. *J. Cell Biol.* **106**, 575–584.

Fernandez, D., Dang, T.A.T., Spudich, G.M., Zhou, X.-R., Berger, B.R. and Christie, P.J. (1996) The *Agrobacterium tumefaciens* VirB7 gene product, a proposed component of the T-complex transport apparatus,

is a membrane-associated lipoprotein exposed at the periplasmic surface. *J. Bacteriol.* **178**, 3156–3167.

Filichkin, S.A. and Gelvin, S.B. (1993) Formation of a putative relaxation intermediate during T-DNA processing directed by the *Agrobacterium tumefaciens* VirD1,D2 endonuclease. *Mol. Microbiol.* **8**, 915–926.

Firth, N., Ippen-Ihler, K. and Skurray, R. (1996) Structure and function of the F factor and mechanism of conjugation. In *Escherichia Coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F.C., ed.), pp. 2377–2401. Washington, DC: American Society for Microbiology.

Forbes, D.J. (1992) Structure and function of the nuclear pore complex. *Annu. Rev. Cell Biol.* **8**, 495–527.

Frolov, M.V., Benevolenskaya, E.V. and Birchler, J.A. (1998) Regena (Rga), a *Drosophila* homolog of the global negative transcriptional regulator CDC36 (NOT2) from yeast, modifies gene expression and suppresses position effect variegation. *Genetics*, **148**, 317–329.

Fullner, K.J., Lara, J.C. and Nester, E.W. (1996) Pilus assembly by *Agrobacterium* T-DNA transfer genes. *Science*, **273**, 1107–1109.

Gaudin, V., Vrain, T. and Jouanin, L. (1994) Bacterial genes modifying hormonal balances in plants. *Plant Physiol. Biochem.* **32**, 11–29.

Gelvin, S.B. (1998) *Agrobacterium* VirE2 proteins can form a complex with T strands in the plant cytoplasm. *J. Bacteriol.* **180**, 4300–4302.

Gielen, J., Terryn, N., Villarroel, R. and Van Montagu, M. (1999) Complete nucleotide sequence of the T-DNA region of the plant tumour-inducing *Agrobacterium tumefaciens* Ti plasmid pTiC58. *J. Exp. Bot.* **50**, 1421–1422.

Gietl, C., Koukolikova-Nicola, Z. and Hohn, B. (1987) Mobilization of T-DNA from *Agrobacterium* to plant cells involves a protein that binds single-stranded DNA. *Proc. Natl Acad. Sci. USA*, **84**, 9006–9010.

Guralnick, B., Thomsen, G. and Citovsky, V. (1996) Transport of DNA into the nuclei of *Xenopus* oocytes by a modified VirE2 protein of *Agrobacterium*. *Plant Cell*, **8**, 363–373.

Heath, J.D., Boulton, M.I., Raineri, D.M., Doty, S.L., Mushegian, A.R., Charles, T.C., Davies, J.W. and Nester, E.W. (1997) Discrete regions of the sensor protein virA determine the strain-specific ability of *Agrobacterium* to agroinfect maize. *Mol. Plant-Microbe Interact.* **10**, 221–227.

Herrera-Estrella, A., Chen, Z., Van Montagu, M. and Wang, K. (1988) VirD proteins of *Agrobacterium tumefaciens* are required for the formation of a covalent DNA protein complex at the 5′ terminus of T-strand molecules. *EMBO J.* **7**, 4055–4062.

Herrera-Estrella, A., Van Montagu, M. and Wang, K. (1990) A bacterial peptide acting as a plant nuclear targeting signal: the amino-terminal portion of *Agrobacterium virD2* protein directs a β-galactosidase fusion protein into tobacco nuclei. *Proc. Natl Acad. Sci. USA*, **87**, 9534–9537.

Hooykaas, P.J.J. and Beijersbergen, A.G.M. (1994) The virulence system of *Agrobacterium tumefaciens*. *Annu. Rev. Phytopathol.* **32**, 157–179.

Howard, E.A. and Citovsky, V. (1990) The emerging structure of the *Agrobacterium* T-DNA transfer complex. *Bioessays*, **12**, 103–108.

Howard, E.A., Winsor, B.A., De Vos, G. and Zambryski, P. (1989) Activation of the T-DNA transfer process in *Agrobacterium* results in the generation of a T-strand protein complex: tight association with the 5′ ends of T-strands. *Proc. Natl Acad. Sci. USA*, **86**, 4017–4021.

Howard, E.A., Zupan, J., Citovsky, V. and Zambryski, P. (1992) The VirD2 protein of *A. tumefaciens* contains a C-terminal bipartite nuclear localization signal: implications for nuclear uptake of DNA in plant cells. *Cell*, **68**, 109–118.

Jin, S.G., Prusti, R.K.T.R., Ankenbauer, R.G. and Nester, E.W. (1990a) Phosphorylation of the VirG protein of *Agrobacterium tumefaciens* by the autophosphorylated VirA protein: essential role in biological activity of VirG. *J. Bacteriol.* **172**, 4945–4950.

Jin, S.G., Roitsch, T., Ankenbauer, R.G., Gordon, M.P. and Nester, E.W. (1990b) The VirA protein of *Agrobacterium tumefaciens* is autophosphorylated and is essential for vir gene regulation. *J. Bacteriol.* **172**, 525–530.

Jin, S.G., Roitsch, T., Christie, P.J. and Nester, E.W. (1990c) The regulatory VirG protein specifically binds to a cis-acting regulatory sequence involved in transcriptional activation of *Agrobacterium tumefaciens* virulence genes. *J. Bacteriol.* **172**, 531–537.

Kalogeraki, V.S., Zhu, J., Eberhard, A., Madsen, E.L. and Winans, S.C. (1999) The phenolic vir gene inducer ferulic acid is O-demethylated by the VirH2 protein of an *Agrobacterium tumefaciens* Ti plasmid. *Mol. Microbiol.* **34**, 512–522.

Kanemoto, R.H., Powell, A.T., Akyoshi, D.E., Reiger, D.A., Kerstetter, R.A., Nester, E.W., Hawes, M.C. and Gordon, M.P. (1989) Nucleotide sequence and analysis of the plant-inducible locus *pinF* from *Agrobacterium tumefaciens*. *J. Bacteriol.* **171**, 2506–2512.

Kato, M., Aiba, H., Tate, S., Nishimura, Y. and Mizuno, T. (1989) Location of phosphorylation site and DNA-binding site of a positive regulator, OmpR, involved in activation of the osmoregulatory genes of *Escherichia coli*. *FEBS Lett.* **249**, 168–172.

Koukolikova-Nicola, Z., Raineri, D., Stephens, K., Ramos, C., Tinland, B., Nester, E.W. and Hohn, B. (1993) Genetic analysis of the *virD* operon of *Agrobacterium tumefaciens*: a search for functions involved in transport of T-DNA into the plant cell nucleus and in T-DNA integration. *J. Bacteriol.* **175**, 723–731.

Lai, E.M. and Kado, C.I. (1998) Processed VirB2 is the major subunit of the promiscuous pilus of *Agrobacterium tumefaciens*. *J. Bacteriol.* **180**, 2711–2717.

Lee, K., Dudley, M.W., Hess, K.M., Lynn, D.G., Joerger, R.D. and Binns, A.N. (1992) Mechanism of activation of *Agrobacterium* virulence genes: identification of phenol-binding proteins. *Proc. Natl Acad. Sci. USA*, **89**, 8666–8670.

Lee, L.Y., Gelvin, S.B. and Kado, C.I. (1999) pSa causes oncogenic suppression of *Agrobacterium* by inhibiting VirE2 protein export. *J. Bacteriol.* **181**, 186–196.

Lee, Y.W., Jin, S., Sim, W.S. and Nester, E.W. (1995) Genetic evidence for direct sensing of phenolic compounds by the VirA protein of *Agrobacterium tumefaciens*. *Proc. Natl Acad. Sci. USA*, **92**, 12 245–12 249.

Lee, Y.W., Jin, S., Sim, W.S. and Nester, E.W. (1996) The sensing of plant signal molecules by *Agrobacterium*: genetic evidence for direct recognition of phenolic inducers by the VirA protein. *Gene*, **179**, 83–88.

Matthysse, A.G. (1986) Initial interactions of *Agrobacterium tumefaciens* with plant host cells. *Crit. Rev. Microbiol.* **13**, 281–307.

Meyer, K., Leube, M.P. and Grill, E. (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science*, **264**, 1452–1455.

Mushegian, A.R., Fullner, K.J., Koonin, E.V. and Nester, E.W. (1996) A family of lysozyme-like virulence factors in bacterial pathogens of plants and animals. *Proc. Natl Acad. Sci. USA*, **93**, 7321–7326.

Mysore, K.S., Bassuner, B., Deng, X.B., Darbinian, N.S., Motchoulski, A., Ream, W. and Gelvin, S.B. (1998) Role of the *Agrobacterium tumefaciens* VirD2 protein in T-DNA transfer and integration. *Mol. Plant-Microbe Interact.* **11**, 668–683.

Mysore, K.S., Kumar, C.T. and Gelvin, S.B. (2000a) *Arabidopsis* ecotypes and mutants that are recalcitrant to *Agrobacterium* root transformation are susceptible to germ-line transformation. *Plant J.* **21**, 9–16.

Mysore, K.S., Nam, J. and Gelvin, S.B. (2000b) An *Arabidopsis* histone H2A mutant is deficient in *Agrobacterium* T-DNA integration. *Proc. Natl Acad. Sci. USA*, **97**, 948–953.

Nakielny, S. and Dreyfuss, G. (1999) Transport of proteins and RNAs in and out of the nucleus. *Cell*, **99**, 677–690.

Nam, J., Matthysse, A.G. and Gelvin, S.B. (1997) Differences in susceptibility of *Arabidopsis* ecotypes to crown gall disease may result from a deficiency in T-DNA integration. *Plant Cell*, **9**, 317–333.

Nam, J., Mysore, K.S., Zheng, C., Knue, M.K., Matthysse, A.G. and Gelvin, S.B. (1999) Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium*. *Mol. Gen. Genet.* **261**, 429–438.

Narasimhulu, S.B., Deng, X.-B., Sarria, R. and Gelvin, S.B. (1996) Early transcription of *Agrobacterium* T-DNA genes in tobacco and maize. *Plant Cell*, **8**, 873–886.

Ninfa, A.J. and Magasanik, B. (1986) Covalent modification of the glnG product, NRI, by the glnL product, NRII, regulates the transcription of the glnALG operon in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **83**, 5909–5913.

Otten, L., DeGreve, H., Leemans, J., Hain, R., Hooykass, P. and Schell, J. (1984) Restoration of virulence of *vir* region mutants of *A. tumefaciens* strain B6S3 by coinfection with normal and mutant *Agrobacterium* strains. *Mol. Gen. Genet.* **195**, 159–163.

Pansegrau, W., Schoumacher, F., Hohn, B. and Lanka, E. (1993) Site-specific cleavage and joining of single-stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: analogy to bacterial conjugation. *Proc. Natl Acad. Sci. USA*, **90**, 11 538–11 542.

Paulsson, M. and Wadstrom, T. (1990) Vitronectin and type-I collagen binding by *Staphylococcus aureus* and coagulase-negative streptococci. *FEMS Microbiol. Immunol.* **65**, 55–62.

Pemberton, L.F., Rosenblum, J.S. and Blobel, G. (1999) Nuclear import of the TATA-binding protein: mediation by the karyopherin Kap114p and a possible mechanism for intranuclear targeting. *J. Cell Biol.* **145**, 1407–1417.

Peng, W.T., Lee, Y.W. and Nester, E.W. (1998) The phenolic recognition profiles of the *Agrobacterium tumefaciens* VirA protein are broadened by a high level of the sugar binding protein ChvE. *J. Bacteriol.* **180**, 5632–5638.

Pionnat, S., Keller, H., Hericher, D., Bettachini, A., Dessaux, Y.X.N. and Poncet, C. (1999) Ti plasmids from *Agrobacterium* characterize rootstock clones that initiated a spread of crown gall disease in Mediterranean countries. *Appl. Environ. Microbiol.* **65**, 4197–4206.

Pollard, T.D. and Cooper, J.A. (1986) Actin and actin-binding proteins. A critical evaluation of mechanisms and functions. *Annu. Rev. Biochem.* **55**, 987–1035.

Rhee, Y., Gurel, F., Gafni, Y., Dingwall, C. and Citovsky, V. (2000) A genetic system for detection of protein nuclear import and export. *Nature Biotechnol.* **18**, 433–437.

Rossi, L., Hohn, B. and Tinland, B. (1993) The VirD2 protein of *Agrobacterium tumefaciens* carries nuclear localization signals important for transfer of T-DNA to plant. *Mol. Gen. Genet.* **239**, 345–353.

Rossi, L., Hohn, B. and Tinland, B. (1996) Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium tumefaciens*. *Proc. Natl Acad. Sci. USA*, **93**, 126–130.

Salmond, G.P.C. (1994) Secretion of extracellular virulence factors by plant pathogenic bacteria. *Annu. Rev. Phytopathol.* **32**, 181–200.

Scheiffele, P., Pansegrau, W. and Lanka, E. (1995) Initiation of *Agrobacterium tumefaciens* T-DNA processing. *J. Biol. Chem.* **270**, 1269–1276.

Shaw, C.H., Watson, M.D., Carter, G.H. and Shaw, C.H. (1984) The right hand copy of the nopaline Ti-plasmid 25 bp repeat is required for tumour formation. *Nucl. Acids Res.* **12**, 6031–6041.

Sheng, J. and Citovsky, V. (1996) *Agrobacterium*–plant cell interaction: have virulence proteins—will travel. *Plant Cell*, **8**, 1699–1710.

Shimoda, N., Toyoda-Yamamoto, A., Aoki, S. and Machida, Y. (1993) Genetic evidence for an interaction between the VirA sensor protein and the ChvE sugar-binding protein of *Agrobacterium*. *J. Biol. Chem.* **268**, 26 552–26 558.

Shirasu, K. and Kado, C.I. (1993) Membrane location of the Ti plasmid VirB proteins involved in the biosynthesis of a pilin-like conjugative structure on *Agrobacterium tumefaciens*. *FEMS Microbiol. Lett.* **111**, 287–294.

Shirasu, K., Koukolikova-Nicola, Z., Hohn, B. and Kado, C.I. (1994) An inner-membrane-associated virulence protein essential for T-DNA transfer from *Agrobacterium tumefaciens* to plants exhibits ATPase activity and similarities to conjugative transfer genes. *Mol. Microbiol.* **11**, 581–588.

Shurvinton, C.E., Hodges, L. and Ream, W. (1992) A nuclear localization signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. *Proc. Natl Acad. Sci. USA*, **89**, 11837–11841.

Spudich, G.M., Fernandez, D., Zhou, X.R. and Christie, P.J. (1996) Intermolecular disulfide bonds stabilize VirB7 homodimers and VirB7/VirB9 heterodimers during biogenesis of the *Agrobacterium tumefaciens* T-complex transport apparatus. *Proc. Natl Acad. Sci. USA*, **93**, 7512–7517.

Stachel, S.E., Messens, E., Van Montagu, M. and Zambryski, P. (1985) Identification of the signal molecules produced by wounded plant cell that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature*, **318**, 624–629.

Stachel, S.E., Timmerman, B. and Zambryski, P. (1986) Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer for *Agrobacterium tumefaciens* to plant cells. *Nature*, **322**, 706–712.

Stachel, S.E., Timmerman, B. and Zambryski, P. (1987) Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement for 5′ *virD* gene products. *EMBO J.* **6**, 857–863.

Stachel, S.E. and Zambryski, P. (1986) *virA* and *virG* control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. *Cell*, **46**, 325–333.

Stachel, S.E. and Zambryski, P. (1989) Bacteria–yeast conjugation. Generic trans-kingdom sex. *Nature*, **340**, 190–191.

Stock, J.B., Stock, A.M. and Mottonen, J.M. (1990) Signal transduction in bacteria. *Nature*, **344**, 395–400.

Sundberg, C., Meek, L., Carrol, K., Das, A. and Ream, W. (1996) VirE1 protein mediates export of single-stranded DNA binding protein VirE2 from *Agrobacterium tumefaciens* into plant cells. *J. Bacteriol.* **178**, 1207–1212.

Sundberg, C.D. and Ream, W. (1999) The *Agrobacterium tumefaciens* chaperone-like protein, VirE1, interacts with VirE2 at domains required for single-stranded DNA binding and cooperative interaction. *J. Bacteriol.* **181**, 6850–6855.

Suzuki, K., Hattori, Y., Uraji, M., Ohta, N., Iwata, K., Murata, K., Kato, A. and Yoshida, K. (2000) Complete nucleotide sequence of a plant tumor-inducing Ti plasmid. *Gene*, **242**, 331–336.

Tinland, B., Koukolikova-Nicola, Z., Hall, M.N. and Hohn, B. (1992)

The T-DNA-linked VirD2 protein contains two distinct nuclear localization signals. *Proc. Natl Acad. Sci. USA*, **89**, 7442–7446.

Tinland, B., Schoumacher, F., Gloeckler, V., Bravo-Angel, A.M. and Hohn, B. (1995) The *Agrobacterium tumefaciens* virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. *EMBO J.* **14**, 3585–3595.

Turk, S.C., van Lange, R.P., Regensburg-Tuink, T.J. and Hooykaas, P.J. (1994) Localization of the VirA domain involved in acetosyringone-mediated vir gene induction in *Agrobacterium tumefaciens*. *Plant Mol. Biol.* **25**, 899–907.

Wagner, V.T. and Matthysse, A.G. (1992) Involvement of vitronectin-like protein in attachment of *Agrobacterium tumefaciens* to carrot suspension culture cells. *J. Bacteriol.* **174**, 5999–6003.

Wang, K., Herrera-Estrella, L., Van Montagu, M. and Zambryski, P. (1984) Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. *Cell*, **38**, 455–462.

Wang, K., Stachel, S.E., Timmerman, B., Van Montagu, M. and Zambryski, P. (1987) Site-specific nick occurs within the 25 bp transfer promoting border sequence following induction of vir gene expression in *Agrobacterium tumefaciens*. *Science*, **235**, 587–591.

Ward, E. and Barnes, W. (1988) VirD2 protein of *Agrobacterium tumefaciens* very tightly linked to the 5′ end of T-strand DNA. *Science*, **242**, 927–930.

Winans, S.C., Mantis, N.J., Chen, C.Y., Chang, C.H. and Han, D.C. (1994) Host recognition by the VirA, VirG two-component regulatory proteins of *Agrobacterium tumefaciens*. *Res. Microbiol.* **145**, 461–473.

Young, C. and Nester, E.W. (1988) Association of the VirD2 protein with the 5′ end of T-strands in *Agrobacterium tumefaciens*. *J. Bacteriol.* **170**, 3367–3374.

Zambryski, P. (1989) *Agrobacterium*–plant cell DNA transfer. In *Mobile DNA* (Berg, D.E. and Howe, M.M., eds). Washington, D.C.: American Society for Microbiology, pp. 309–333.

Zambryski, P. (1992) Chronicles from the *Agrobacterium*–plant cell DNA transfer story. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 465–490.

Zambryski, P., Depicker, A., Kruger, K. and Goodman, H.M. (1982) Tumor induction by *Agrobacterium tumefaciens*: analysis of the boundaries of T-DNA. *J. Mol. Appl. Genet.* **1**, 361–370.

Zambryski, P., Tempe, J. and Schell, J. (1989) Transfer and function of T-DNA genes from *Agrobacterium* Ti and Ri plasmids in plants. *Cell*, **56**, 193–201.

Zhou, X.R. and Christie, P.J. (1999) Mutagenesis of the *Agrobacterium* VirE2 single-stranded DNA-binding protein identifies regions required for self-association and interaction with VirE1 and a permissive site for hybrid protein construction. *J. Bacteriol.* **181**, 4342–4352.

Zhu, J.-K., Damsz, B., Kononowicz, A.K., Bressan, R.A. and Hasegawa, P.M. (1994) A higher plant extracellular vitronectin-like adhesion protein is related to the translational elongation factor-1α. *Plant Cell*, **6**, 393–404.

Ziemienowicz, A., Gorlich, D., Lanka, E., Hohn, B. and Rossi, L. (1999) Import of DNA into mammalian nuclei by proteins originating from a plant pathogenic bacterium. *Proc. Natl Acad. Sci. USA*, **96**, 3729–3733.

Zupan, J., Citovsky, V. and Zambryski, P. (1996) *Agrobacterium* VirE2 protein mediates nuclear uptake of ssDNA in plant cells. *Proc. Natl Acad. Sci. USA*, **93**, 2392–2397.

Zupan, J., Ward, D. and Zambryski, P. (1998) Assembly of the VirB transport complex for DNA transfer from *Agrobacterium tumefaciens* to plant cells. *Curr. Opin. Microbiol.* **1**, 649–655.

Zupan, J. and Zambryski, P. (1997) The *Agrobacterium* DNA transfer complex. *Crit. Rev. Plant Sci.* **16**, 279–295.