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Update on Plant Transformation

Transfer of T-DNA from *Agrobacterium* to the Plant Cell[1]

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Agrobacterium tumefaciens is the causative agent of crown gall, a disease of dicotyledonous plants characterized by a tumorous phenotype. Earlier in this century, scientific interest in *A. tumefaciens* was based on the possibility that the study of plant tumors might reveal mechanisms that were also operating in animal neoplasia. In the recent past, the tumorous growth was shown to result from the expression of genes coded for by a DNA segment of bacterial origin that was transferred and became stably integrated into the plant genome. This initial molecular characterization of the infection process suggested that *Agrobacterium* might be used to deliver genetic material into plants. The potential to genetically engineer plants generated renewed interest in the study of *A. tumefaciens*. In this review, we concentrate on the most recent advances in the study of *Agrobacterium*-mediated gene transfer, its relationship to conjugation, DNA processing and transport, and nuclear targeting. In the following discussion, references for earlier work can be found in more comprehensive reviews (Hooykaas and Schilperoort, 1992; Zambryski, 1992; Hooykaas and Beijersbergen, 1994).

OVERVIEW OF *AGROBACTERIUM*-MEDIATED GENE TRANSFER

A. tumefaciens is exploited by many plant biologists in molecular and genetic studies to introduce DNA into plants. Although best known for this practical application, the transfer of DNA from bacterium to plant comprises fundamental biological processes, many of which are largely uncharacterized.

During infection by *A. tumefaciens*, a piece of DNA is transferred from the bacterium to the plant cell (Fig. 1). This piece of DNA is a copy of a segment called the T-DNA (transferred DNA). It is carried on a specific plasmid, the Ti-plasmid (tumor-inducing), which is found in only a small percentage of natural soil populations of *A. tumefaciens*. The T-DNA is delimited by 25-bp direct repeats that flank the T-DNA. These borders are the only *cis* elements necessary to direct T-DNA processing. Any DNA between these borders will be transferred to a plant cell. In contrast to other mobile DNA elements, T-DNA does not itself encode the products that mediate its transfer. Wild-type T-DNAs encode enzymes for the synthesis of the plant growth regulators auxin and cytokinin and the production of these compounds in transformed plant cells results in the tumorous phenotype. In addition, wild-type T-DNA also encodes enzymes for the synthesis of novel amino acid derivatives called opines. The Ti-plasmid encodes enzymes for their catabolism; hence, *Agrobacterium* has evolved to genetically commandeer plant cells and use them to produce compounds that they uniquely can utilize as a carbon/nitrogen source. Additional profit derives from the ability of opines to stimulate conjugation of the Ti-plasmid, thereby increasing the bacterial population that can utilize opines (reviewed by Greene and Zambryski, 1993).

The processing and transfer of T-DNA are mediated by products encoded by the *vir* (virulence) region, which is also resident on the Ti-plasmid (Stachel and Nester, 1986). Those *vir* genes, whose products are directly involved in T-DNA processing and transfer, are tightly regulated so that expression occurs only in the presence of wounded plant cells, the targets of infection. Control of gene expression is mediated by the VirA and VirG proteins, a two-component regulatory system (reviewed by Winans, 1992). VirA detects the small phenolic compounds released by wounded plants resulting in autophosphorylation (Fig. 1, step 1). VirA phosphorylation of VirG then leads to activation of *vir* gene transcription.

Following *vir* gene induction, the production of a transfer intermediate begins with the generation of the T-strand, an ss copy of the T-DNA (Stachel et al., 1986). VirD1 and VirD2 are essential for this process (Filichkin and Gelvin, 1993). Together, VirD1/D2 recognize the 25-bp border sequence and produce an ss endonucleolytic cleavage in the bottom strand of each border (Fig. 1, step 2) These nicks are used as the initiation and termination sites for T-strand production. After nicking, VirD2 remains tightly associated with the 5′ end of the T-strand. The lone VirD2 at the 5′ end gives the T-complex a polar character that may ensure that, in subsequent steps, the 5′ end is the leading end. T-strand production is thought to result from the displacement of the bottom strand of the T-DNA between the nicks. Recent work has suggested that the early steps in T-strand production are evolutionarily related to other bacterial systems that produce ssDNA, such as during conjugation (Table I; discussed below).

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Abbreviations: ds, double-stranded; NLS, nuclear localization signal; ss, single-stranded.

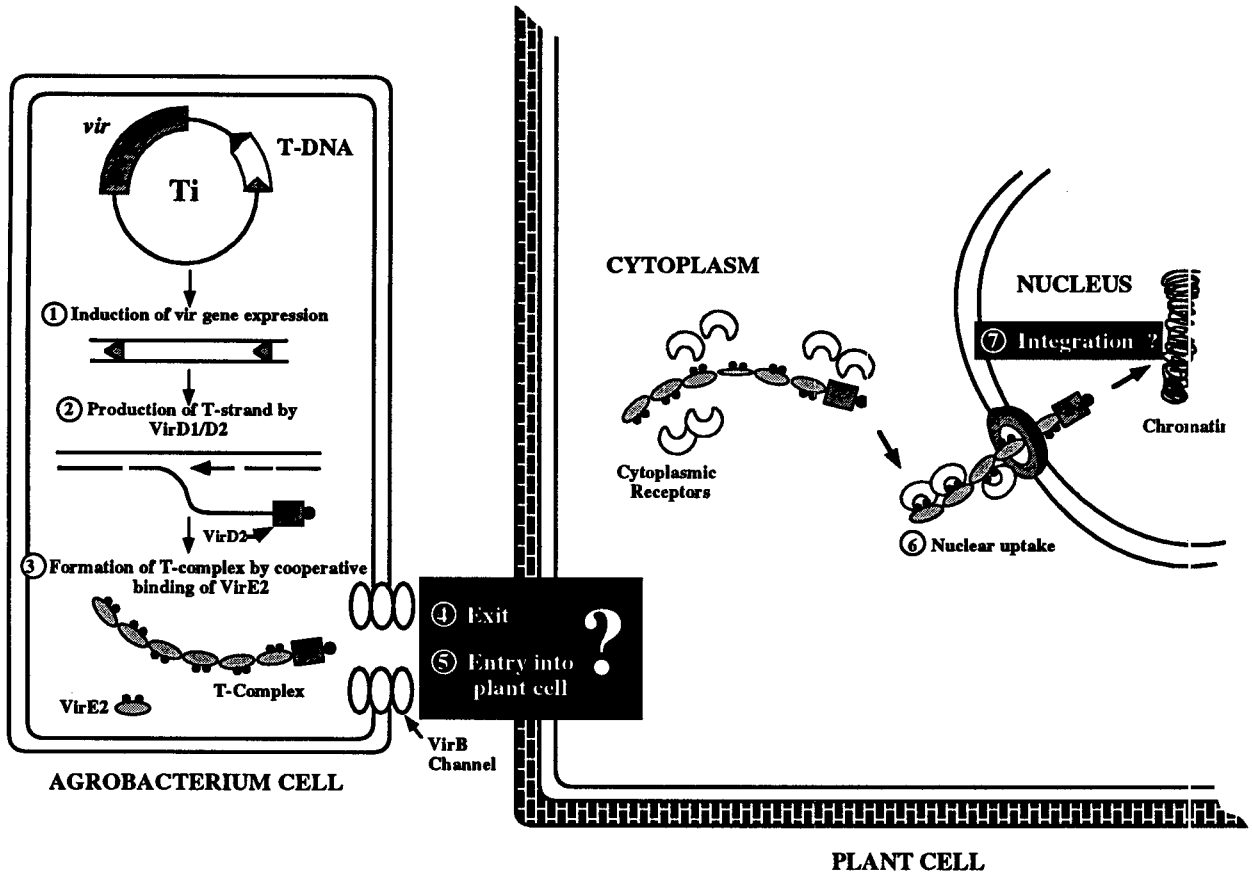

Figure 1. Basic steps in the transformation of plant cells by *A. tumefaciens*. Each step is described in the text.

The T-strand must travel through numerous membranes and cellular spaces before arrival in the plant nucleus. Thus, to preserve its integrity, it was hypothesized that the T-strand likely travels as an ssDNA-protein complex. VirE2 is an inducible ss nucleic acid-binding protein encoded by the *vir*E locus that binds without sequence specificity. VirE2 binds tightly and cooperatively, which means that a T-strand would be completely coated (Fig. 1, step 3). Consequently, degradation by nucleases would be prevented and, indeed, in vitro binding of VirE2 renders ssDNA resistant to nucleolytic degradation. Finally, binding of VirE2 unfolds and extends ssDNA to a narrow diameter of 2 nm, which may facilitate transfer through membrane channels. The T-strand along with VirD2 and VirE2 are termed the T-complex.

Subsequently, the T-complex must exit the bacterial cell (Fig. 1, step 4), passing through the inner and outer membranes as well as the bacterial cell wall. It must then cross the plant cell wall and membrane (Fig. 1, step 5). Once inside the plant cell, the T-complex targets to the plant cell nucleus and crosses the nuclear membrane (Fig. 1, step 6), after which the T-strand becomes integrated into a plant chromosome (Fig. 1, step 7). In the context of experimental chronology and relevant results, steps 1 through 3 (Fig. 1) have been well studied. Current and recent research has related to steps 4 and 6. Entering the plant cell (step 5) and the mechanics of integration (step 7) are almost completely uncharacterized.

RELATIONSHIP OF T-DNA TRANSFER TO CONJUGATIVE DNA TRANSFER

Evidence supporting the hypothesis that *Agrobacterium*-plant DNA transfer and bacterial conjugative DNA transfer are evolutionarily related has been rapidly accumulating. This hypothesis was first proposed following the discovery of the T-strand (Stachel and Zambryski, 1986; Stachel et al., 1986) and was based on striking parallels with the synthesis and transfer of conjugal DNA. First, short sequences required in *cis* (*ori*T and T-DNA right border) are functionally polar in directing DNA transfer. Transfer is initiated by nicks in these sequences, and reversing their orientation inhibits DNA transfer. Second, ss linear DNA is transferred following its displacement from the plasmid. Finally, DNA transfer will occur only if the donor and recipient cell are in intimate contact.

Recent studies have not only confirmed these functional analogies but extended them to include similarities in the amino acid sequences, gene organization, and physical properties of the *trans*-acting enzymes involved. Functional and molecular similarities of T-DNA transfer and conjugation are most completely characterized with respect to the

Table I. *Comparison of the similarities between T-DNA transfer and bacterial conjugative DNA transfer in the RP4 system*

Key elements of each system are in bold and elements with nucleotide or amino acid sequence similarity are underlined.

Characteristic	Proposed Analogy between RP4 and T-DNA Transfer
Initiation site	DNA transfer initiates at the **right border** (T-DNA) and at the **nick region** of *ori*T (RP4); see text for sequence similarity.
Endonuclease assembly	**VirD1,D2** form a complex on the right border (T-DNA) and **TraI,J** along with TraH assemble at the nick site (RP4).
Nicking	Site-specific DNA cleavage by **VirD2** (T-DNA) and **TraI** (RP4), both of which remain attached to the 5′ end of the DNA to be transferred.
Transfer intermediate	**T-strand**, ssT-DNA copy, is released from Ti-plasmid by an unknown mechanism followed by the cooperative binding of **VirE2**, an ssDNA-binding protein, to form **T-complex**. Transferred ssDNA is unwound from RP4; no conjugation-specific, ssDNA-binding protein has been identified.
Transfer	T-DNA transfer and ss conjugative DNA transfer (RP4) occurs directionally from 5′ to 3′.
Export	Products of **VirB** and **Tra2** operons primarily form structure for export of DNA from donor and may have a role in import of transferred DNA into recipient.

plasmid RP4, a broad-host-range plasmid originally isolated from a strain of *Pseudomonas* (Table I). The study of RP4 and T-DNA transfer has been mutually informative (reviewed by Lessl and Lanka, 1994). First, the development of in vitro techniques for the study of DNA processing in the RP4 system has led directly to the biochemical characterization of some Vir proteins. Second, comparative analysis of the RP4 Tra2 operon, involved in transfer of DNA from donor to recipient, and the *Agrobacterium vir*B operon has suggested possible roles for some of the proteins encoded by these operons in DNA transfer.

Processing of the Transfer Intermediate in *Agrobacterium* and RP4

DNA transfer begins with an ss nick at specific sites in both the T-DNA and the conjugative DNA in RP4. In vivo, TraI and TraJ cooperate as the RP4 relaxase to nick *ori*T. In *Agrobacterium*, VirD1/D2 cleave the T-DNA border sequences between the third and fourth bases. Both nick sites contain a 12-nucleotide consensus sequence (AC/TC/AT/CATCCTGC/TC/A) that has also been found among various rolling circle-type replicons (Waters and Guiney, 1993; Lessl and Lanka, 1994). The *ori*T of RSF1010, the IncQ conjugative plasmid, and its cognate relaxase (mobilization) proteins actually can substitute for T-DNA borders in the transfer of DNA to a plant cell.

In vitro, TraI and VirD2 are each sufficient, in the presence of Mg^{2+}, to produce nicks in ss oligonucleotides bearing their respective cognate nick sites (Pansegrau et al., 1993; refs. in Lessl and Lanka, 1994). In the presence of an excess of cleavage products, both TraI and VirD2 can also catalyze the opposite reaction, joining two pieces of ssDNA. It is curious that VirD2 can catalyze the cleavage of *ori*T in an ss oligonucleotide but TraI cannot cleave a ssT-DNA right border. The ability to recognize other nick sites is consistent with the hypothesis that VirD2 initiates the integration of T-strand by joining it to the 3′ side of an ss nick in plant DNA. Consequently, if it also binds to a plant DNA site prior to joining, VirD2 may have evolved to tolerate more variability in the DNA sequences to which it will bind. If the cognate sites are present in supercoiled ds plasmids, TraJ and VirD1 must be included with TraI and VirD2, respectively, to obtain nicking. Neither complex will cleave its cognate nick site if it is present in the form of a relaxed ds circle or a linear double strand.

At the molecular level, TraI has three highly conserved motifs at the N terminus that it shares with VirD2 and relaxases of other conjugative systems (Pansegrau et al., 1994). Motif I contains a Tyr at position 22 in TraI that forms a phosphodiester between its aromatic hydroxyl group and the 5′ phosphoryl group of the DNA during cleavage (Pansegrau et al., 1993). Motif III in TraI contains two His's necessary for cleavage that may serve to activate the Tyr and for coordinate binding of Mg^{2+}. Recognition of the binding site 3′ to the nick may be the role of motif II. Critical amino acids for these functions have been identified in TraI by mutagenesis and are predicted to be conserved in VirD2 based on sequence comparison. The Tyr to which the transferred ssDNA becomes attached occurs at position 29 in VirD2. This amino acid is not only within a context equivalent to that around position 22 in TraI but also cannot be altered without abolishing nicking activity.

The role of VirD1 may be deduced by comparison to TraJ, its presumed counterpart in the RP4 system. Previously, bacterial extracts enriched in VirD1 were suggested to have topoisomerase I activity. This does not, however, appear to be an enzymatic activity of purified VirD1. In RP4, TraJ first binds to the 3′ side of *ori*T and then recruits TraI. This sequence of events appears to be specific to the nicking of supercoiled dsDNA. By analogy, VirD1 may first recognize and bind the T-DNA border, which promotes VirD2 binding.

Export from Bacterium

Export of the T-complex from *Agrobacterium* is one of the steps in the transfer process about which very little is known. The steps in generating a transferable T-DNA copy were linked to one or more of the *vir* operons. Export, however, was a function without an operon. Early work showed that *vir*B was necessary for tumorigenesis but was not required for T-strand or T-complex formation (Stachel and Nester, 1986). Sequencing of *vir*B suggested that its gene products may be involved in the transfer process (reviewed by Zambryski, 1992). The 11 open reading frames of the *vir*B locus code for proteins that exhibit the predicted features necessary for forming a membrane-associated export apparatus, including hydrophobicity, membrane-spanning domains, and/or N-terminal signal sequences that target protein out of the cytoplasm.

Subcellular localization studies confirmed the membrane association of seven VirB proteins (Thorstenson et al., 1993; Beijersbergen et al., 1994; Thorstenson and Zambryski, 1994). All seven proteins, however, were distributed between the inner and outer membranes, in proportions that varied depending on the fractionation method (Thorstenson et al., 1993). It has been suggested that these VirB proteins are so tightly juxtaposed in a complex that spans both membranes that they fractionate randomly between the inner and outer membranes.

By sequence comparison, *vir*B has been shown to be evolutionarily related to other bacterial gene clusters whose products form a membrane-associated complex involved in fimbriae formation, protein secretion, or DNA uptake (reviewed by Lessl and Lanka, 1994). Comparison of a genetic element of RP4, the Tra2 region, to *vir*B has shed light on the process of DNA transfer in these two systems. The Tra2 region of RP4 encodes 11 proteins involved in the DNA-transfer step of conjugation, probably forming the pilus or pilus precursors. Six of the Tra2 proteins are highly similar to VirB proteins, especially in features that suggest membrane localization, e.g. hydrophobic domains (Lessl et al., 1992). The genes for five of these Tra2 proteins are collinear with their *vir*B counterparts. At least two of these proteins, VirB2 and TrbC, show some sequence similarity to TraA (from *Escherichia coli* F factor), a bona fide pilus subunit.

Two VirB proteins, VirB4 and 11, are candidates to provide the energy for translocation of the T-complex. Both localize to the inner membrane, consistent with this proposed function. VirB4 has a nucleotide-binding site that is essential for virulence (Berger and Christie, 1993). VirB11 is reported to associate with the cytoplasmic face of the inner membrane, and it exhibits both ATPase and protein kinase activity. VirB11 is related by amino acid sequence to TrbB of the Tra2 operon, which possesses similar enzymatic activities. Proteins similar to TrbB are found in a variety of bacteria usually in membrane-associated, multi-protein complexes that function in import or export of protein and DNA. Lessl and Lanka (1994) suggested that TrbB-like proteins may form a class of ATPases that function specifically in complexes that translocate macromolecules and macromolecular complexes across the bacterial inner and outer membranes.

The *vir*B operon also appears to be closely related to the Ptl operon (Weiss et al., 1993). The Ptl operon (*Bordetella pertussis*) encodes products responsible for export of pertussis toxin protein. Five of the encoded proteins are significantly similar, and four of the genes for these proteins are collinear. Within TrbB-like proteins, VirB11 and TrbB form a closely related subgroup that also includes PtlH. Therefore, the machinery to export the pertussis toxin may have evolved from the same progenitor as the systems to transfer DNA between bacteria and from bacterium to plant. Possibly the transport systems for T-DNA and conjugation evolved from a protein export system and now recognize only the protein component of the transfer intermediates. The T-strand, essentially masked by VirD2 and VirE2, may be coincidentally carried along as transport is mediated by protein-protein interactions (Thorstenson and Zambryski, 1994).

Aside from a requirement for activated VirG to induce transcription, little is known about additional, more subtle mechanisms that may regulate *vir*B expression. The *vir*B operon is transcribed as a single polycistronic message that includes all 11 open reading frames, and most mutagenesis strategies tend to be polar, which requires that the *vir*B operon be reconstructed to express genes downstream of the one being tested. Thus, assessing expression requirements and the necessity of individual loci for virulence is extremely labor intensive in *vir*B. Recent, in-depth studies, however, have shown *vir*B2 through *vir*B11 to be absolutely required for virulence and *vir*B1 mutants severely attenuated in virulence (Dale et al., 1993; Berger and Christie, 1994).

Expression of several *vir*B genes depends on *cis*-acting elements. Mutants for these *vir*B loci were not restored to wild-type virulence unless the complementing plasmid included adjacent sequences or entire adjacent genes (Dale et al., 1993; Berger and Christie, 1994). These requirements for adjacent genes can be partially explained by the proposed translational coupling between *vir*B1/2 and *vir*B8/9. Accumulation of *vir*B9 was shown to be much reduced in both *vir*B7 and *vir*B8 mutants (Berger and Christie, 1994). By analogy to P pilus formation, in which coordinate protein synthesis can stabilize individual proteins through protein-protein interaction (Kuehn et al., 1993), the accumulation of some VirB proteins may require other VirB proteins. An interaction with another protein(s) may promote either stability or proper localization before degradation. In fact, both accumulation and localization of VirB3 are dependent on VirB4 (Jones et al., 1994). *Agrobacterium* that did not express VirB4 accumulated greatly reduced cellular levels of VirB3, which fractionated with the inner membrane. In the presence of VirB4, wild-type levels of VirB3 appeared in both the inner and outer membrane fractions.

Almost nothing is known about the interaction(s) between the putative VirB complex and T-complex presumed to occur prior to and during export. VirD4 has been suggested to mediate these interactions based on its requirement for virulence and its inner membrane localization

(Thorstenson and Zambryski, 1994). VirD4 is not required for T-strand or T-complex formation. TraG is the VirD4 counterpart in the RP4 system. Because TraG is required for conjugation but is involved in neither nick-site cleavage or pilus formation, it has been speculated to be involved in linking the mobilized DNA to the pilus during transfer. The functional analogy between VirD4 and TraG is supported by the demonstration that VirD4 and the VirB proteins can substitute for TraG and Tra2 products, respectively, in mediating the transfer of RSF1010 between *Agrobacteria*.

All evidence to date supports the hypothesis that *vir*B is involved in the transfer of the T-complex out of the bacterium. Sequence data and comparison to conjugative DNA transfer systems indicate that at least some of the *vir*B products form a membrane-associated complex that may be functionally similar to a pilus. The remaining VirB proteins may assist in assembly of the complex or provide ancillary functions during T-complex transfer (e.g. ATPase activity for energy).

SS INTERMEDIATE

Although the early evidence for the T-strand intermediate was compelling, the debate about what is the "real" T-DNA copy has nevertheless continued for the last 10 years. Evidence for ds circle as well as ds linear molecules has been presented (reviewed by Zambryski, 1992). Both of these ds forms, however, are found only in the bacterium and probably result from the nicks in the 25-bp repeats, either promoting recombination to generate circles or promoting nonspecific opposite-strand breakage during DNA extraction. In two recent papers, however, the debate has hopefully been put to rest. Two very different strategies were used to assay the nature of the T-DNA copy upon arrival in the plant cell. In both cases, the results most strongly supported an ss transfer intermediate.

Yusibov et al. (1994) monitored (a) the synthesis and disappearance of the T-DNA copy within *Agrobacterium* and (b) the appearance of a PCR-detected T-DNA segment in tobacco protoplasts. The disappearance of the T-DNA copy from *Agrobacterium* was correlated with the appearance in the plant cytoplasmic fraction of the T-DNA segment. The latter product was not amplified if the plant cytoplasmic fraction was first treated with S1 nuclease that possessed no detectable ds DNase activity. Control experiments excluded the possibility that lysed bacteria were the source of the T-DNA template. The lack of a PCR product after nuclease S1 treatment supports the hypothesis that the transfer intermediate is indeed an ss molecule.

In the second study, a sensitive extrachromosomal recombination assay was used (Tinland et al., 1994). Briefly, the strategy was to design a T-DNA with a GUS gene divided into two overlapping segments. In planta recombination is then required to yield a full-length gene. Extrachromosomal recombination that takes place early after entry of the T-complex into the nucleus can discriminate between the transfer of dsDNA and ssDNA. If the transfer intermediate is ds, recombination should produce an intact GUS regardless of whether the segments in the T-DNA are of the same or opposite polarity. If the transfer intermediate is ss, however, then a complete GUS can be obtained through recombination only if the segments are of opposite polarity. GUS activity in infected protoplasts was an order of magnitude greater from the T-DNA bearing GUS segments of opposite polarity relative to GUS segments of the same polarity. Thus, this study and that by Yusibov et al. (1994) provide strong confirmation for an ss transfer intermediate.

NUCLEAR IMPORT OF T-COMPLEX

Integration of T-strand requires nuclear uptake, which is a tightly regulated process that can occur only through the nuclear pore. Proteins larger than approximately 40 kD require an NLS that mediates their nuclear uptake. The T-complex has three components that could potentially contribute the signal(s) for nuclear import. The T-strand itself is unlikely to possess a signal for nuclear import, since any DNA between borders will be transferred. Nuclear transport of T-complex is most likely mediated by its associated proteins, VirD2 or VirE2.

Sequence analysis of VirD2 revealed that the N terminus is 85% conserved among strains of *Agrobacterium*; the C terminus is only 25% conserved, but the highest similarity is in the last 30 amino acids (reviewed by Zambryski, 1992). Within this domain, a sequence homologous to the bipartite type of NLS was found (Howard et al., 1992). The nuclear localizing function of this sequence was confirmed by fusing its coding region to the GUS reporter gene and transiently expressing this construct in tobacco protoplasts (Howard et al., 1992). The nuclear localization of the fusion protein was inferred from the accumulation of the blue GUS product in the nucleus. When the putative NLS was deleted from full-length VirD2 fused to GUS, the GUS product was only in the cytoplasm (Howard et al., 1992). Nuclear accumulation of transiently expressed VirD2 has also been demonstrated by immunolocalization (Tinland et al., 1992). That the C-terminal bipartite NLS sequence has a relevant biological function was confirmed by the observation that *Agrobacterium* was severely reduced in tumorigenicity when the two basic stretches of the bipartite NLS were deleted from VirD2 (Shurvinton et al., 1992).

Although VirD2 possesses a functional NLS necessary to mediate nuclear uptake of T-complex, the T-complex is an extremely large nucleoprotein complex (reviewed by Zambryski, 1992). In some strains of *Agrobacterium*, the T-strand is 20 kb. This T-strand would bind approximately 600 molecules of VirE2 and would have a calculated length of 3600 nm, 60 times the thickness of the nuclear membrane. Including a single VirD2, the T-complex has a mass of about 50×10^6 D. Is a single bipartite NLS capable of mediating the nuclear uptake of a complex this large?

If additional NLSs are required for nuclear uptake, they may be supplied by VirE2, the most abundant protein component of the T-complex. Sequence analysis revealed two potential bipartite NLSs in VirE2. As with VirD2, the nuclear localization of VirE2 was tested by fusing its coding regions to GUS. Although mutants in which one or the other of the NLSs were deleted exhibited some nuclear

transport, both NLSs were required for maximum accumulation in the nucleus (Citovsky et al., 1992). Thus, VirE2 provides NLSs along the entire length of the T-complex. The multiple NLSs of VirE2 may be functionally important for uninterrupted nuclear import of T-complex, e.g. keeping the cytoplasmic and nucleoplasmic sides of the nuclear pore open simultaneously.

The presence of NLSs in VirE2 raises the question of whether the NLS of VirD2 is superfluous or has a unique role in the import of T-complex. That *Agrobacterium* bearing an NLS-deleted *vir*D2 was severely attenuated in virulence suggests the latter. Possibly, VirD2 initiates uptake and ensures that the 5′ end of the T-strand enters the nucleus first. Leading with the 5′ end may be a common feature of the translocation of ss nucleic acids across the nuclear pore. For example, export of the 75S rRNA (Balbioni Rings) from the nuclei of salivary gland cells in *Chironomus tentans* initiates at the 5′ end (Mehlin et al., 1992).

Compelling evidence for an in planta role for VirE2 is derived from experiments using wild type or *vir*E2 mutants of *Agrobacterium* in combination with transgenic tobacco plants expressing wild-type or mutant VirE2. In the first set of experiments, avirulent *vir*E mutants of *Agrobacterium* were restored to wild-type virulence when inoculated on tobacco expressing wild-type VirE2 (Citovsky et al., 1992). Complementation was reduced on plants expressing transgenic VirE2 in which either NLS had been deleted (Citovsky et al., 1992). Potentially, two functions might be complemented in planta, nuclear import or protection from nucleases, because mutagenesis of either NLS also reduced ss nucleic acid-binding activity in vitro. In the second set of experiments, wild-type VirE2-expressing transgenic tobacco was partially resistant to infection by a virulent (i.e. VirE2$^+$) strain of *Agrobacterium* (Citovsky et al., 1994). Resistance was maximal in plants expressing mutant VirE2 that was unable to bind ssDNA but still contained both NLSs. Thus, nuclear import is at least one of the in planta functions that might be inhibited by VirE2 expressed in transgenic plants (Citovsky et al., 1994).

The recalcitrance of monocots to transformation by *Agrobacterium* is a puzzle that remains unsolved. Although considered unlikely given the evolutionary conservation of bipartite NLSs from plants to animals, nuclear localization was one of the steps at which a block could possibly occur. Both VirD2 and VirE2 in fusion to GUS, however, accumulated in the nuclei when transiently expressed in epidermal cells of maize leaves and immature sections of the root (Citovsky et al., 1994). Thus, both of the *Agrobacterium* proteins associated with the T-complex are recognized by the nuclear import machinery of a monocot cell, which suggests that the complex itself could be imported into the nucleus.

Clearly, the study of *Agrobacterium* has led to discoveries in other areas of biology, from plant-bacterial signaling to conjugation to nuclear import. Most recently, the study of nuclear import in the process of T-complex transfer unexpectedly revealed a possible developmental component to the regulation of nuclear import (Citovsky et al., 1994). Transiently expressed VirD2 and VirE2 GUS fusions accumulated in the nuclei of leaf and immature root epidermal cells of both tobacco and maize. In mature root epidermis, however, both fusion proteins remained cytoplasmic. It may be that nuclear import of VirD2 and VirE2 is mediated by NLS-binding proteins that are not produced in all cell types. Developmentally specific gene expression could potentially be achieved by a cell type-specific complement of NLS-binding proteins. Different subsets of transcription factors would be admitted to the nucleus, depending on which NLS-binding protein(s) were expressed in that cell type. Increasingly, nuclear import regulation is being shown to have a role in the control of transcription factor activity (Whiteside and Goodbourn, 1993). Further evidence of multiple pathways for nuclear import is indicated by transgenic tobacco expressing VirE2. Nuclear import associated with normal cellular processes is apparently not significantly inhibited by the transgenic protein (Citovsky et al., 1994).

Integration of foreign DNA into a host chromosome is the final step of the T-DNA transfer process (Fig. 1, step 7) and it is this function, unique among plant pathogens, that is largely responsible for the intense investigation of *Agrobacterium*. However, the mechanics of this step remain largely unknown. The best model to date compares T-DNA integration to illegitimate recombination (Gheysen et al., 1991). It is suggested that the VirD2-bound 5′ end of the T-strand joins a nick in plant DNA. The plant DNA may further unwind to form a gap, and the 3′ end of the T-strand may pair with another region of plant DNA close by. Plant repair and recombination enzymes may then function to covalently join the 3′ end to plant DNA. These reactions would result in the introduction of the T-strand into one strand of plant DNA. Torsional strain would then result in the introduction of a nick into the opposite strand of plant DNA. Gap repair and DNA synthesis using the T-strand as template result in the final integration product.

FUTURE DIRECTIONS

A. tumefaciens is the only known naturally occurring organism capable of genetically transforming a plant cell. It is both a tool for the plant molecular biologist and a reservoir of fascinating biology. Although some of the processes whereby DNA is transferred from *Agrobacterium* to a plant are understood in some detail, others are not. The relationship between *vir*B products and T-complex export is still unresolved. Although an export-specific channel is the predicted *vir*B product, nothing is known about the details of its assembly, structure, or function. In conjugative DNA transfer, the pilus, which in some bacteria is a product of an operon similar to *vir*B, has a role in contacting the recipient cell. Whether any of the *vir*B products has a role in (pilus type) recognition of the plant cell surface is not known. The binding of *Agrobacterium* to the plant cell surface is another point in the infection process at which monocots may be resistant. Monocots are known to produce inducers of *vir* gene expression and, as discussed above, are competent to import T-complex into the nucleus. Thus, it may be that proteins on the surface of *Agrobacterium* are unable to recognize receptors on the surface of the monocot cell,

thereby preventing attachment and transfer of T-DNA. Finally, nothing is known about the enzymology of integration. No plant factors have been implicated in this process. VirD2 is presumed to catalyze the integration of T-strand into the plant genome; however, no data have been obtained to support this hypothesis. It is also not known whether VirE2 plays a role in this process, possibly performing a function analogous to that of RecA (West, 1992). Future research should address these questions and may, as in the past, shed considerable light on other areas of biology.

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