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Pathways of DNA transfer to plants from *Agrobacterium tumefaciens* and related bacterial species

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Abstract

Genetic transformation of host plants by *Agrobacterium tumefaciens* and related species represents a unique model for natural horizontal gene transfer. Almost five decades of studying the molecular interactions between *Agrobacterium* and its host cells have yielded countless fundamental insights into bacterial and plant biology, even though several steps of the DNA transfer process remain poorly understood. *Agrobacterium* spp. may utilizs different pathways for transfer of its DNA, which likely reflects the very wide host range of *Agrobacterium*. Moreover, closely related bacterial species, such as rhizobia, become able transfer DNA to host plant cells when they are provided with *Agrobacterium* DNA transfer machinery and T-DNA. Homologs of *Agrobacterium* virulence genes are found in many bacterial genomes, but only one non-*Agrobacterium* bacterial strain, *Rhizobium etli* CFN42, harbors a complete set of virulence genes and can mediate plant genetic transformation when carrying a T-DNA-containing plasmid.

INTRODUCTION

Agrobacterium tumefaciens is often described as a natural genetic engineer, equipped to horizontally transfer bacterial genes and genetically transform plant cells (45). Indeed, transfer of genetic material from *A. tumefaciens*, and related species, to their host plants represents the first known case of active horizontal gene transfer from prokarya to eukarya. The main factors conferring this ability to *A. tumefaciens* are located on the large Ti (tumor inducing) plasmid, which contains a region with the *vir* (virulence) genes encoding most of the proteins required to mediate the DNA transfer, and the T-DNA (transferred DNA) itself. The T-DNA sequences naturally transferred by several *Agrobacterium* spp. contain two types of genes, under the control of promoters compatible with expression in eukaryotic cells. The first set of genes ("oncogenes") encodes proteins that affect biosynthesis of or plant cell response to growth regulators (auxins and cytokinins) and induce uncontrolled cellular division, resulting in tissue proliferation and formation of neoplastic growths (crown-galls). The second set of genes encodes enzymes involved in the synthesis of small molecules (opines), composed of an amino acid and an organic acid or a carbohydrate, which can be used by *Agrobacterium* cells as source of carbon and nitrogen (36). The ability of *Agrobacterium* to transfer DNA, either for transient expression or for stable genetic transformation, is widely used as a tool in research and biotechnology (11). Whereas the mechanism of T-DNA transfer and integration has been studied extensively from the early 1970s, there are still many aspects of the process that are not completely understood. Recent discoveries indicate that there is a potentially important variability in the pathways used by *Agrobacterium* strains to deliver the T-DNA to the plant genome, which may also reflect adaptation to different hosts. Furthermore, the presence of homologs of the *Agrobacterium* genes

required for virulence in related bacterial species suggests that DNA transfer to eukaryotes might be more widespread among bacteria outside the *Agrobacterium* genus.

MECHANISM OF *AGROBACTERIUM*-MEDIATED DNA TRANSFER AND INTEGRATION

A. tumefaciens interactions with host plant cells represent a reference model for the transfer of DNA from bacteria to eukaryotic cell. The molecular mechanism of T-DNA transfer from *Agrobacterium* to its host plant cell genome has been reviewed in detail in several articles (45; 65); here, we will provide a brief account of the current state of knowledge of this system. Several *Agrobacterium* species are known to genetically transform plants, resulting in distinct plant diseases (36); however, most of the research on the mechanism of DNA transfer has focused on a few strains of *A. tumefaciens* (i.e., the nopaline C58 and octopine A6 strains), which are presented in this section. For the purpose of this review, we divided the process of transfer of DNA from *A. tumefaciens* to its host cell genome into four steps (see Fig. 1 for an overview).

Step 1. Virulence induction and generation of single-stranded T-DNA

Signal recognition and integration

Agrobacterium cells can detect several plant-emitted signals and respond by modifying their lifestyle and adjusting the transcription level of their *vir* genes. The key regulator of *vir* gene expression is the two-component receptor system encoded by the *vir*A and *vir*G genes (117). The first-identified and major plant-produced molecule involved in *vir* activation is acetosyringone (3,5-dimethoxyacetophenone, AS), a phenolic compound often found in plant

exudates; AS activates the VirA/VirG two-component system, resulting in the induction of the *vir* gene expression (17; 115). Whereas *vir*A and *vir*G are expressed at low levels without induction, they are themselves inducible by AS (138), and VirA/VirG activation results in rapid and strong induction of all the *vir* genes. Reducing sugar monomers, such as D-glucose and D-galactose, can both increase the sensitivity of VirA/VirG system to phenolics and elevate the saturating concentration of phenolics for virulence activation (21; 113). These monosaccharides bind to the periplasmic chromosome-encoded protein ChvE, which then interacts with the VirA periplasmic domain to enhance its *vir* gene-inducing activity (21; 112). VirA is also activated by low pH (between pH 5 and 6), either directly (86) or via its interaction with ChvE (41). In addition, low pH combined with low phosphate concentration activates a different two-component regulatory system (ChvG/ChvI), which results in increased expression of *vir*G (24). The response to low pH relies on the periplasmic ChvG inhibitor ExoR, which is degraded under acidic conditions (52).

Activating vir *gene expression*

Upon activation of VirA/VirG system, VirG is phosphorylated and activates *vir* gene expression by direct binding to a 10 to 12-bp sequence (*vir* box) (118). One or several *vir* boxes are found in promoter regions of each of the *vir* operons, usually located between 50 and 200 bp upstream of the translation initiation of the first gene of each *vir* operon. Expression of *vir* promoters is observed a few hours after the initial induction by phenolics, and it generally reaches a plateau after 12 to 24 hours (138). In addition to several signaling pathways that converge to generate the activated (phosphorylated) VirG, there are other factors affecting *vir* gene expression. For example, the *vir*C and *vir*D operons are repressed by Ros, a chromosome-

encoded transcriptional regulator (30). More recently, small RNAs regulating some of the *vir* genes were identified (34).

Turning off vir *gene expression*

After successful infection of a host plant by *Agrobacterium* cells, i.e., when the virulence system is no longer needed, the virulence system should be shut off. Indeed, the energy cost of virulence induction is high, and the activation of virulence results in a decrease of the population growth rate (100). Among potential factors negatively affecting *vir* gene expression, IAA (indole acetic acid) interferes with *vir* gene induction, probably as a competitive inhibitor of AS binding to VirA (78). The role of IAA, synthesized at high levels during the development of *Agrobacterium*-induced crown gall tumors, could be to turn off virulence induction as well as to inhibit additional transformation by competing bacterial strains or by the initially infecting strain. In addition, the change of lifestyle of the bacteria, between free-living, non-pathogenic bacteria and pathogenic cells attached to the plant cell surface and embedded in the biofilm matrix, may also affect the *vir* gene expression.

Generation of single-stranded T-DNA

The T-DNA is a segment of the Ti-plasmid, delimited by two short (24-25 bp) direct repeat sequences, the left (LB) and right borders (RB) (99; 141). T-DNA is mobilized from the Ti plasmid and transferred into the host cell as a single-stranded DNA (ssDNA) intermediate, termed the T-strand (116). Two essential proteins for T-DNA processing are VirD1 and VirD2. VirD2 is an endonuclease (2; 144), which, in association with the VirD1 DNA topoisomerase (47), mediates the mobilization of the transferrable T-DNA from the Ti-plasmid via a strand

replacement mechanism. Importantly, the T-DNA borders are the only sequences required for recognition by VirD2/VirD1, and, thus, the sequences between these borders may be modified at will. Two other Vir proteins, VirC1 and VirC2, have been shown to increase the number of T-strand molecules, most likely by binding to sequences, termed overdrive, close to the T-DNA borders (33). At the end of the process, VirD2 remains covalently linked to the 5' end of the T-strand (145), forming an "immature" T-complex.

Step 2. Export of the T-DNA and effector proteins and cell-to-cell interactions

Macromolecules are translocated across the bacterial membranes via a T4SS (type four secretion system), composed of the 11 proteins encoded by the *vir*B operon and VirD4, via a mechanism closely resembling plasmid transfer during bacterial conjugation. T4SSs are molecular complexes that mediate the transport of proteins or nucleoprotein complexes, usually comprising an ssDNA with a protein molecule at its 5'-end, through the membranes and cell wall of gram-negative bacteria (27).

Targeting of exported macromolecules to T4SS

Interactions with bacterial factors are likely required to mediate targeting of the exported substrates—i.e., the VirD2-T-strand complex and the effector proteins VirD5, VirE2, VirE3, and VirF—to the VirB/D4 T4SS. Protein export from *Agrobacterium* may occur independently of DNA export, and it depends on the presence of an arginine-rich export signal found in all exported Vir proteins (130; 131). Several factors have been suggested to mediate targeting of the VirD2-T-strand complex and individual translocated proteins to the T4SS machinery. For example, VirC1 and VirC2 may assist targeting of VirD2 and the T-strand to the bacterial membrane at the cell poles where T4SS is thought to be assembled (7). VirE2 might be recruited

to the cell poles via its association with the coupling protein VirD4 (8). Other bacterial factors, VBPs (VirD2 binding proteins), appear important for VirD2 recruitment to T4SS, as well as for recruitment of diverse relaxase proteins in other T4SS systems (51). Indeed, VBPs, conserved in *Agrobacterium* spp., can target VirD2 and the associated T-strand to the energizing components of the T4SS, i.e., VirD4, VirB4 and VirB11, and, thus, to the T-DNA export machinery (49; 51). Furthermore, VBPs, which do not interact with the other exported effector proteins, appear to be important for recruitment of conjugative DNA transfer intermediates to T4SS during conjugation (51).

For the most part, the transport pathway of the VirD2-T-strand complex through bacterial membranes has been deciphered, and it comprises sequential interactions with different protein components of T4SS (23). The lumen size of the VirB2 pilus of T4SS appears sufficient to accommodate passage of ssDNA and partially unfolded proteins (62); indeed, in other bacterial species, relaxases transported through T4SS channels have been reported to unfold during transport (121).

Attachment and close-range cell-to-cell interactions

Close-range interaction and attachment between bacterial and host cells is thought to be required for transfer of T-DNA and effector proteins (83). Whereas, under laboratory conditions, bacterial virulence can be induced without interaction with the host cell surface, in nature these two events are likely linked, and the induction of virulence is coincidental with a change in the bacterial cell lifestyle, from free bacteria in the rhizosphere to bacteria attached at the surface of the host cell and embedded in a biofilm. Indeed, the same signals that trigger *vir* gene expression also induce chemotaxis. Specifically, *Agrobacterium* cells respond to phenolics and sugars

secreted by plants by moving toward their source via chemotaxis (50); at low concentration of these molecules chemotaxis is activated while their high concentrations result in virulence activation. Moreover, in addition to its positive effect on virulence, low phosphate concentration enhances biofilm formation and cellular adhesion (140).

By analogy to other host-plant interactions, such as *Rhizobium*-legume symbiosis (103), the cellular interaction is believed to occur in two steps. The initial contact between bacteria and eukaryotic cells usually relies on host cell-surface receptors and represents a reversible interaction. Then, the bacterial attachment is stabilized, via the synthesis of cellulose fibrils, and bacterial cells are embedded in a biofilm at the surface of the plant tissue. In the case of *Agrobacterium*, the precise role of the different factors affecting attachment during the infection process is not completely understood. The *Agrobacterium* T4SS components, i.e., VirB2 (pilin) and VirB5, exposed to the bacterial cell surface, represent good candidates for interaction with potential host cell receptors (9). Four *Arabidopsis* proteins were identified to interact with VirB2 (59), and shown to affect the efficiency of the T-DNA transfer. However, it is not clear whether these VirB2-interacting proteins are involved in cellular attachment or in other steps of the DNA transfer process, such as signal transmission or passage of the T-DNA through the host-cell membrane. Interestingly, pilin homologs encoded by *Agrobacterium*, CtpA and PilA, appear to be involved in the early stages of *Agrobacterium* cell surface attachment, although their role in virulence remains unknown (135). VirB5 localizes at the tip of the VirB2 pilus (4) and may have a dual function: first - during T4SS biogenesis, which requires VirB5 expression in the bacterial cell, and second - outside the bacterial cell (64), although it plays no obvious role in cellular attachment. Exocellular polysaccharides produced by *Agrobacterium* also play a role in attachment and biofilm formation. Synthesis and export of cyclic 1,2-ß-D-glucan, which relies

on proteins encoded by the *chv*A, *chv*B, and *exo*C genes, is involved in attachment and virulence (22; 32); UPPs (unipolar polysaccharides) and cellulose also may play a role in bacterial adhesion and biofilm formation (82; 140). However, plant receptors that, similarly to the plant lectins facilitating *Rhizobium*-plant cell recognition (53), bind these exopolysaccharides and are involved in *Agrobacterium*-mediated transformation have not been identified.

Step 3. Entry and subcellular sorting of T-DNA and effector proteins in the host cell

Entry of T-DNA and associated proteins into the host cell cytoplasm

The mechanism by which the VirD2-T-strand complex and effector proteins pass through the host cell wall and plasma membrane is unknown. Although wounding of the plant tissue enhances the *Agrobacterium* mediated transformation efficiency, T-DNA transfer from *Agrobacterium* without wounding of the host plant cell has been reported (18). Several mechanisms are possible for entry through the host plasma membrane. First, similarly to a mechanism proposed for bacterial conjugation, depolymerization of the VirB2 pilus may bring the bacterial outer membrane and the host cell plasma membrane together, resulting in temporary membrane fusion and allowing the transfer of cargo (20). Second, the VirB2 pilus may act as a "needle", via a mechanism similar to T3SS-mediated effector protein transport (93); in this scenario, macromolecular substrates pass through the pilus, and the pilus interacts with the host membrane or integral membrane proteins to allow the entry of the cargo. So far, however, no interactions with host membrane proteins or bacterial factors able to form a pore in the host membrane have been identified in the *Agrobacterium*-host plant cell system. Moreover, *Agrobacterium* mutants unable to form pili still retain a low-level virulence, demonstrating that T-DNA transfer can occur in the absence of the VirB2 pilus (105). Third, macromolecules could

be first exported into the intercellular space and then internalized by the host cell, for example, via an endocytosis-like mechanism, which might involve recognition between the exported macromolecules and a potential host receptor. Indeed, a recent study suggested that VirE2 associates with early endosomes in the host plant cell, and that endocytosis inhibitors affected both VirE2 transport and transformation efficiency (75). Thus, endocytosis might be involved in the internalization of VirE2 and potentially of other translocated molecules. In addition, VirE2 has been shown to form channels through artificial membranes (35); although formation of VirE2 pores has not been demonstrated in infected plant cells, such pores might mediate transport of other macromolecules through the host cell membranes.

Nuclear import

Before integration can occur, the T-DNA, as well as translocated effector proteins with a nuclear function, must be imported into the nucleus. Efficient nuclear import via simple diffusion is unlikely for large molecules such as T-strands. Genetic transformation of plant cells using protocols that do not involve *Agrobacterium* implies that nuclear import of foreign DNA can occur without exogenous effector proteins, most likely using cellular DNA binding proteins that facilitate import; such transformation techniques are considered less efficient than the *Agrobacterium*-mediated transformation, although it is difficult to compare efficiency between such different methods. The nuclear import step can be circumvented altogether if the transformation process occurs during cell division, when the nuclear envelope is disrupted (132). However, transient expression of T-DNA, which obviously requires its nuclear import, occurs efficiently in non-dividing cells following agroinfiltration (143). Thus, active nuclear import most likely is involved in most cases of *Agrobacterium*-mediated genetic transformation.

Generally, bacterial proteins interacting with the T-DNA are presumed to mediate its nuclear uptake the importin alpha-mediated import pathway. First VirD2, attached to the 5'-end of the T-DNA, interacts directly with importin alpha via its NLS sequences and is targeted to the host cell nucleus (10). VirE2, an ssDNA binding protein, is also thought to interact with the T-DNA after its entry in the host cell cytoplasm, forming the mature T-complex (28; 44). Whereas the VirE2-T-strand complex has not been shown directly to form in living cells, a significant amount of data suggests that such formation occurs. First, the T-DNA integrated in absence of VirE2 displays increased truncations, suggesting that VirE2 associates with and protects the T-strand against degradation (104). Then, VirE2 has a strong affinity for ssDNA in vitro (26; 28), producing helical ssDNA-VirE2 filaments with well-defined structure (1). Initially, several studies demonstrated that VirE2 tagged with different markers was targeted to the nucleus in plant cells, at least partially (29; 77; 148). Other studies showed that fusion of VirE2 with fluorescent proteins remained largely cytoplasmic (72; 110). Because of its strong homopolymerization, VirE2 tends to form aggregates when expressed ectopically in plant cells, which hinders assessment of its localization; it is also possible that only a fraction of VirE2 is directed to the nucleus, but that this fraction is sufficient for VirE2 functionality in the T-strand import process. VirE2 was shown to interact with several plant proteins likely to affect its intracellular distribution and/or function: VIP1 (123), VIP2 (5), importins alpha (14), and core histones (68; 80). Moreover, VirE2 also interacts with VirE3 (70; 76), and this interaction likely assists accumulation of VirE2 at the sites of entry into the host cell (76) and/or subsequent nuclear import of VirE2 (70). Both VirD2 and VirE2 have been shown to mediate nuclear import of short segments of ssDNA (149). Potentially, these two proteins participate in T-DNA nuclear import; VirD2, alone or with the help of VirE2, targets the T-strand to the nuclear pores while

VirE2 packages the T-strand and mediates its movement through host cell cytoplasm (149) and through the nuclear pore. It has also been suggested that T-strand and its associated proteins could interact with the host cell cytoskeleton and ER during its transport to the nucleus (142).

Step 4: T-DNA integration in the host chromosomal DNA

The mechanism of T-DNA integration into the host genome remains largely obscure (46). We will first present the main known facts about the integration process and then incorporate them into potential integration pathways. Two main approaches have been used to characterize T-DNA integration: analysis of the locations of the integrated T-DNA and its patterns of integration, and studies of plant and bacterial factors that may affect integration. In the first approach, early studies showed that integrated T-DNAs were preferentially located in transcriptionally active chromatin (3); however, these studies relied on analyses of transgenic plants regenerated under antibiotic selection, which made it virtually impossible to detect integration into heterochromatin which does not support expression of the antibiotic resistant reporter. Indeed, a completely different result was obtained in studies performed without selection, which showed that T-DNA integrated randomly in all regions of chromatin (63), although a local bias might occur toward specific epigenetic markers (111). Nucleosomal histones have been suggested to be involved in the targeting of T-DNA complex to the host chromatin, by allowing interaction between the T-complex and the host chromatin before integration. First, histone H2A was found to be important for T-DNA integration, as an *Arabidopsis* mutant in this gene displayed lower transformation efficiency (88), and, later, other histones were shown to increase T-DNA integration (119). Interaction between VIP1 and

different histones was also demonstrated (80), and VirD2 was found to interact with histones (139).

Unlike many integrating viruses, *Agrobacterium* does not encode a dedicated integrase among its effector proteins. Although early studies suggested that VirD2 might act as an integrase (95; 120); later, integration was shown to be mediated by host factors (150). Yet, it cannot be excluded that VirD2 or another *Agrobacterium* translocated effector protein facilitate T-DNA integration by interacting with the host factors that directly mediate integration. Moreover, the analysis of integration in various host species, particularly with different yeast mutants, has shown that the integration of T-DNA relies mostly on the host cell pathways. Several studies have suggested a role for the host cell DNA repair pathways in T-DNA integration, and double-strand DNA breaks (DSBs) were shown to represent preferred sites for T-DNA integration (25; 107; 122). Measuring T-DNA integration rates in *Arabidopsis* mutants in different genes encoding DNA repair proteins yielded inconclusive results (see below). Using a combination of these two approaches, it was recently reported that an *Arabidopsis* mutant in the DNA polymerase theta was deficient in T-DNA integration, suggesting an integration mechanism based on micro-homologies (129). DNA polymerase theta was first identified as a suppressor of genome instability, and it is known for its role in genomic DNA ligation in the microhomology-mediated end-joining (MMEJ, or alternative end-joining, alt-EJ) DSB repair pathway (16). However, this mechanism does not explain integration of double-stranded T-DNA and recombination between several T-DNAs in different orientation, suggesting that several concurrent integration pathways may underlie transformation events (46).

Potential pathways for T-DNA integration

T-DNA enters the nucleus as a segment of ssDNA; it may either be converted to a double-stranded DNA (dsDNA) before integration, most likely into a DSB in the genomic DNA, or anneal partially to the host genomic DNA via micro-homologies before synthesis of its second strand and ligation. There is direct proof that T-DNA can integrate into DSBs as a dsDNA; by introducing a rare cutting dsDNA endonuclease site in both the T-DNA and the host genome and transiently expressing this enzyme, precise reconstruction of the original restriction site at junctions between T-DNA and host DNA was observed (25; 122). Interestingly, it has been shown that the formation of circularized T-DNA (T-circles) occurs after T-DNA transfer into the plant cell (114), although there is no indication that these T-circles act as substrate for integration. The observation of micro-homologies at the junction of some integration sites suggests that the second pathway is also possible, and recent implication of DNA polymerase theta in T-DNA integration (129) shows involvement of this pathway in integration.

Experiments using T-DNA transfer into yeast (*Saccharomyces cerevisiae*)—this model host allows the use of numerous viable mutants in different DSB repair pathways—demonstrated that the integration pathway depends mostly on the host mechanisms. Taking advantage of the ability of yeast cells to support DNA integration via either homologous recombination (HR) or non-homologous end joining (NHEJ), depending on the presence of homologous sequences in the target genome and in the T-DNA, T-DNA integration was assessed in mutants impaired in these pathways. Disruption of Rad52 or Rad51 resulted in integration only via NHEJ while, in the absence of Ku70 or Mre11 expression, only integration via HR was observed (127; 128). In plants, HR occurs only at extremely low rates (48; 84), and NHEJ is believed to be the main pathway for foreign DNA integration. However, studies using *Arabidopsis* mutants in the NHEJ pathways yielded conflicting results. AtLig4 and AtKu80 were found to be dispensable for T-

DNA integration in one study (40), but were required in two other studies (39; 74). More recently, a systematic survey of *Arabidopsis* mutants impaired in different genes involved in the known pathways of NHEJ, reported that T-DNA integration efficiency was not reduced in any of these lines, and it was even increased in some of the mutants (97). In rice, however, reduced rates of overall integration were observed in plant lines with down-regulated Ku70, Ku80 and Lig4 (90). Due to high levels of redundancy between DNA repair pathways, it is difficult to prove their specific involvement in T-DNA integration. Yet, when several NHEJ pathways were mutated in *Arabidopsis*, the resulting viable plants supported only very low levels of T-DNA integration (87). The involvement of DSB repair pathways in T-DNA integration also appears to be complex and may vary at different time points during the infection process. For example, targeting of the incoming T-DNA to "open" DSBs may be achieved in a less efficient repair pathway, but subsequent ligation of the T-DNA into the DSB may require efficient DSB repair. Moreover, the host NHEJ machinery may be manipulated by *Agrobacterium* effector proteins; for example, VirE2 interacted with XRCC4, a component of the NHEJ pathway, and potentially prevented DSB repair, allowing the T-DNA to be targeted to an available DSB site (126). Other host nuclear proteins, such as the transcriptional regulator VIP2 (VirE2 interacting protein 2), might play a role in T-DNA integration (5). Finally, histone post-translational modification (specifically, methylation) was shown to affect T-DNA integration (60).

VARIABILITY OF MOLECULAR PATHWAY FOR *AGROBACTERIUM* INFECTION

Different strains and species of *Agrobacterium* use different pathways for transfer of DNA to different eukaryotic organisms. Besides the wide range of plant species that serve as hosts to *Agrobacterium* spp. in nature (31), under artificial conditions this range extends further

to species from all the clades of the plant kingdom (89) as well as to non-plant cells, such as yeast, other fungi, and animal cultured cells (69/ and reference therein). This possibility to transfer DNA to virtually all eukaryotic cell types (69) reflects *Agrobacterium* adaptability beyond plant-specific factors.

Essential and optional virulence-associated genes

Agrobacterium's virulence factors fall into three main categories. First, are the core factors absolutely essential for T-DNA transfer: the two-component regulatory system (VirA, VirG), the T-strand processing machinery (VirD1, VirD2), T4SS (VirB1-VirB11, VirD4). Second, are the factors important, but not absolutely essential, such that in their absence the T-DNA transfer occurs only at very low efficiency: VirE2 effector, VirC1, VirC2. Third, are the factors non-essential overall, but likely with a role in determination of host range and/or in further facilitating infection, for example, in competition with other microorganisms: the effector proteins VirD3, VirD5, VirE3, VirF; some bacterial strains contain additional Vir proteins that fall into this non-essential category, such as VirH, VirJ, VirK, VirL, VirM. Several proteins, usually termed Chv, encoded by the bacterial chromosome also play an important role in *Agrobacterium* interactions with plant cells; they are involved in different steps of infection, such as virulence activation (ChvE, ChvG, ChvI, ChvH) or cellular adhesion and biofilm formation (ChvB, ChvA, ExoC). Finally, in addition to the Ti plasmid, some *Agrobacterium* strains carry a large At plasmid; its function appears not essential for DNA transfer, although it encodes factors with activities related to survival in the competitive rhizosphere environment, such as quorum sensing mechanisms that regulate plasmid exchange in bacterial populations (101). Whereas all species and strains of *Agrobacterium* share a common general mechanism for T-DNA transfer,

there is a certain degree of variability between them, which translates into differences in the bacterial virulence factors and affects the outcome of infection. As described above, the transfer of T-DNA by *Agrobacterium* relies on core of essential factors. Presumably, the function of these proteins is conserved between different virulent *Agrobacterium* strains, although they may interact with different host factors. Non-essential genes defined based on virulence of the corresponding mutants in highly susceptible hosts, such as tobacco or kalanchoe (57). Thus, although they are not absolutely required for transformation of these plants, they may be necessary to infect other plant species or they may provide a competitive advantage to achieve successful infection in nature.

Variability in inducers and repressors of the vir *genes*

The outcome of the interactions between a specific *Agrobacterium* strain and its specific host plant is also affected by the signal molecules emitted by the host and by the response of the bacteria to these signals. Perception of inducing signals varies between different *Agrobacterium* strains, which may reflect adaptation to specific hosts. A wide variety of phenolic compounds, related to AS, can activate *vir* gene expression (86), including glycoside derivatives (61). Genetic studies identified the protein able to recognize these phenolic compounds as VirA, via swapping *vir*A genes between different strains of *Agrobacterium*, thereby modifying the range of recognized phenolic molecules (73). That different *Agrobacterium* strains show variable responses to different phenolic compounds may confer onto each strain a specific inducibility corresponding to the phenolics emitted by its specific host species. This sensing of phenolics by VirA in different *Agrobacterium* strains may be affected by specific monosaccharides which are sensed by the chromosomal virulence protein ChvE (98).

Other plant-produced molecules also affect *Agrobacterium*'s virulence, likely contributing to the variability of T-DNA transfer efficiencies in different plant species or tissues. Among the signal molecules emitted by plants in response to biotic or abiotic stresses, salicylic acid (SA) inhibits *vir* gene expression, probably by attenuating the VirA protein kinase activity (146). Tobacco or *Arabidopsis* plants overproducing SA or treated with exogenous SA displayed increased resistance to *Agrobacterium* whereas plants deficient in SA accumulation were more sensitive (6; 146). Moreover, ethylene might also inhibit *Agrobacterium*'s virulence, although its direct effect on *vir* gene expression has not been demonstrated (92). Some plant species emit chemicals that inhibit *Agrobacterium* virulence most likely contributing to the variability of susceptibility of different species to *Agrobacterium*. For example, DIMBOA (2,4-dihydroxy-7methoxy-2*H*-1,4-benzuxazin-3(4*H*)-one) and MDIBOA (2-hydroxy-4,7-dimethoxybenzoxazin-3-one), two chemical compounds found in maize homogenates, are strong inhibitors of *Agrobacterium* AS-induced virulence and growth (106).

VirE2 versus GALLS

VirE2 is not absolutely essential for *Agrobacterium*-mediated transformation, although transformation efficiency of *vir*E2 mutants is very low and results in a greater proportion of truncated T-DNA (104). Interestingly, some strains of *A. rhizogenes*, do not contain the *vir*E2 gene but carry the *GALLS* gene instead, which complements *vir*E2 in a *vir*E2-deficient strain of *Agrobacterium*, although its mode of action appears different from VirE2 (54). *GALLS* encodes two proteins, corresponding to the full-length coding sequence or to its C-terminal part, which interact with VirD2 and are targeted to host cell nucleus (55). Their exact function in the transformation process remains unknown.

Interaction of VirE2 with VIP1 and its homologs

The interaction between VirE2 and VIP1 was first discovered via a yeast-two-hybrid screening (123). Using transgenic tobacco over-expressing VIP1 from *Arabidopsis* (AtVIP1), it was shown that VIP1 overexpression increased transformation rate and that VIP1 likely facilitated the nuclear import of VirE2, and thus of the T-complex (123; 124). Other studies, using *Arabidopsis* mutants, however, reached a different conclusion that VIP1 was not required for *Agrobacterium* mediated T-DNA transfer (110). Recently, VirE2 proteins from four different *Agrobacterium* strains were all shown to interact with AtVIP1 and with one or more of its close *Arabidopsis* homologs; interestingly, binding efficiency for the different AtVIP1 homologs was different among the different VirE2 proteins (134). This interaction between VirE2 and several AtVIP1 homologs was confirmed in a more recent paper (71). This study also showed that plants expressing a dominant negative mutant of AtVIP1, i.e., AtVIP1 fused to a SRDX transcriptional repression domain, did not affect transformation; yet SRDX inhibits only the function of AtVIP1 as transcriptional activator, rather than as a VirE2 binding partner. On the other hand, an *Arabidopsis* mutant with three AtVIP1 homologs disrupted showed a modest reduction in transformation efficiency (71), suggesting that functional redundancy might mask the role of these proteins in transformation.

Involvement of ubiquitin/proteasome system, roles of VirF

The VirF effector from the *A. tumefaciens* A6 octopine-type strain (A6-VirF) was shown to contain an F-box domain and to bind several *Arabidopsis* ASK proteins, the Skp1 homologs that function in the SCF pathway for proteasomal degradation (108). The F-box protein activity

of A6-VirF was demonstrated in yeast and in plant cells, and one of its potential substrates and interacting partners was identified; specifically, VirF was shown to bind VIP1 and destabilize via the ubiquitin/proteasom*e* system (UPS) both VIP1 and its associated VirE2 (125). More recently, several other *Arabidopsis* interactors of VirF were identified (42; 43), one of which, VFP4, a transcriptional regulator of genes involved in stress or defense response, was targeted by VirF for proteasomal degradation (42). In addition, VirF itself is destabilized via UPS, and this destabilization is prevented by VirF interaction with another effector, VirD5 (81). Historically, the C58 nopaline-type strain of *A. tumefaciens* has not been considered to encode an active VirF (85), but more recent data suggested that the C58-VirF can bind Arabidopsis ASK proteins via its predicted F-box domain, which suggests that it is a bona fide F-box protein indeed (66). Unlike A6-VirF, C58-VirF did not interact with VIP1, suggesting that it has a different set of targets. Most bacterial species able to transfer DNA (e.g., *A. rhizogenes*, *A. vitis*, and *R. etli*) encode a VirF homolog with potentially different target specificities, likely contributing to the host range specificity of these bacteria. Also, an *Arabidopsis* F-box protein VBF was shown to substitute to A6-VirF in targeting VIP1 for degradation, potentially explaining the dispensability of VirF for infection of this plant species (147). Interestingly, VBF was among the plant genes whose expression is activated by VirE3, which has a transcriptional regulator activity in plants (91).

T-DNA TRANSFER BY NON-*AGROBACTERIUM* BACTERIAL SPECIES

The most common pathogenic *Agrobacterium* species include *A. tumefaciens*, *A. rhizogenes*, and *A. vitis*. The taxonomy of *Rhizobiaceae* is still subject to revision, and we follow the commonly used classification where *Agrobacterium* species are named according to their

pathogenic interactions with plants (37). Their pathogenicity relies on Ti-plasmids, or on Ri-plasmids in the case of *A. rhizogenes*, which are highly diverse and present a mosaic structure (94). Moreover, because these plasmids can be transmitted by conjugation within or even between these bacterial species, other non-*Agrobacterium* species could gain the Ti plasmid features that allow transfer DNA to eukaryotic hosts.

Agrobacterium *T-DNA transfer machinery in related bacterial species*

Introducing the *Agrobacterium vir* region and T-DNA into several species of plant-associated bacteria—pathogenic or symbiotic, nitrogen-fixing—related to *Agrobacterium* spp. has conferred onto these bacteria the ability to genetically transform plants. So far, all such bacterial species have belonged to two families of the Rhizobiales order, *Rhizobiaceae* and *Phyllobacteriaceae*. For example, conjugative transfer of the Ti-plasmid from a virulent *Agrobacterium* strain to *Rhizobium trifolii* resulted in virulent bacterial cells, able to induce crown gall formation in several plant species (56). Several other bacterial species became capable of transforming plants after they have received two plasmids: a helper plasmid, carrying the *Agrobacterium vir* region, and a binary plasmid with an engineered T-DNA. Using this strategy, *Arabidopsis*, tobacco and rice were transformed by three different bacterial species, *Rhizobium leguminosarum*, *R. trifolii* and *Phyllobacterium myrsinacearum* (19) while *Sinorhizobium meliloti*, *Rhizobium* sp. NGR234, and *Mesorhizobium loti* were used to transform potato (137). Similarly, *Ensifer adherens* (syn. *Sinorhizobium adherens*), harboring a cointegrated plasmid, carrying the *vir* region from *Agrobacterium* and an engineered T-DNA, was able to transform potato and rice plants (136; 151). Thus, all these bacterial species likely possess the chromosomally encoded function required for transformation, but not the *vir* gene

functionalities. Attempts to transform plants or other eukaryotes by introducing plasmids carrying a *vir* region and a T-DNA into bacteria outside of the Rhizobiales order, such as *E. coli*, have been unsuccessful (79; 96). *Agrobacterium* spp. are facultative pathogens (12), with a possible transition between pathogenic and non-pathogenic lifestyles. Horizontal gene transfer between bacterial species is well documented (13) via the exchange of plasmids by conjugation; thus, *vir* region-carrying plasmids may be shared among a pool of related bacteria in the rhizosphere, thereby conferring pathogenicity to the recipient cells.

Rhizobium etli *CFN42 contains functional* vir *genes*

Whereas DNA transfer to plants can be achieved using different bacterial species provided with *Agrobacterium*'s *vir* region, these bacterial species do not encode a endogenous complete and functional DNA transfer machinery, making *Agrobacterium* the only species with natural genetic transformation capability. This notion has been altered by the observations that the *Rhizobium etli* CFN42 strain, a symbiotic nitrogen-fixing bacterium associated with host-plants such as beans, contains in its p42a plasmid a complete and functional *vir* region, able to mediate DNA transfer and stable integration into the plant genome, albeit with a low efficiency, when a vector carrying a T-DNA with a reporter gene is provided (67). *R. etli* CFN42 strain with mutated *vir*G or *vir*E2 as well as *R. leguminosarum*, a closely related bacterial strain that does not contain close homologs of the *Agrobacterium vir* genes, were incapable of T-DNA transfer. The *vir* regions of *R. etli* and of *Agrobacterium* share extensive similarity yet exhibit two significant differences: the *R. etli vir*B2 gene is not a part of the *vir*B operon but constitutes a separated operon with its own promoter, and there are two *vir*F operons in *R. etli*. Analysis of *vir* gene expression in *R. etli* showed a pattern of expression close to that observed in

Agrobacterium, notably induction by AS, except for the *vir*B2 gene that was expressed constitutively at low levels and was not induced by AS (133). Interestingly, the *R. etli* p42a plasmid was shown to be exchanged between *R. etli* and related species, including *Agrobacterium* spp. (15). Although *R. etli* has evolved to encode and preserved the functional *vir* machinery, it remains unknown whether this species also contains endogenous T-DNA-like sequences that could be transferred to the plant hosts.

CONCLUSIONS

There is a wide diversity in the pathways underlying each step of DNA transfer from pathogenic *Agrobacterium* and related species. This unique capability relies on a core of essential bacterial factors and on their interactions with different host cell factors. In addition, many other bacterium-encoded proteins represent facultative virulence factors that are not essential for DNA transfer to model plants highly susceptible to *Agrobacterium*. Rather, these non-essential factors may be required for infection of specific hosts as well as for achieving maximally successful infection in the competitive rhizosphere environment. The virulence genes are mostly located on a large plasmid, transmissible between bacterial cells by conjugation, but functions encoded by the bacterial genome are also important for efficient T-DNA transfer under natural conditions. The diversity of pathways, as well as the large array of bacterial factors presumed to facilitate and optimize infection, likely confers to *Agrobacterium* spp. their seemingly unlimited range of host cells under natural or experimental conditions.

Our present knowledge of the *Agrobacterium*-mediated T-DNA transfer raises an interesting question: what constitutes the minimal T-DNA transfer machinery? Among the essential *vir*-encoded proteins, most represent pathways common to many bacterial species. For

example, the *vir*B and *vir*D operons encode a DNA transfer machinery similar to those involved in plasmid transfer by conjugation, and the VirA/VirG sensors regulating expression of *vir* genes is representative of the widespread bacterial two-component regulatory systems. These common pathways may allow easy acquirement by other bacterial species of the genetic transformation capability via acquirement of the functional *vir* region. Such gene transfer possibly occurs within natural bacterial populations, and it would render pathogenic those bacterial species that are not normally considered as such, e.g., rhizobia, usually engaged in symbiotic relationship with their host plants).

Interestingly, by introducing an artificial transferrable DNA into several human pathogens, DNA transfer to cultured human cells was achieved under laboratory conditions (38; 109), in a T4SS dependent manner. Analyses of complete eukaryotic genome sequences, which are becoming increasingly available, have shown that they contain a significant number of sequences originating from prokaryotes and resulting from horizontal gene transfer (58; 67). It makes biological sense that at least some of these sequences had been acquired from bacteria via a mechanism similar to the *Agrobacterium*-mediated T-DNA transfer. In some cases, bacterial sequences present in genomes from several plant species of the *Nicotiana* and *Linaria* genera, as well as sweet potato, can be traced back to their *Agrobacterium*-like donor bacteria that share homologies with today's *Agrobacterium* pathogenic species (reviewed in102).

UNANSWERED QUESTIONS

Some of the fundamental questions about the mechanism of the *Agrobacterium*-mediated T-DNA transfer remain unanswered. How do the T-DNA and its associated proteins pass through the host cell plasma membrane? What is the exact role of VirE2, and of its interacting

plant proteins, in packaging the T-DNA and facilitating T-DNA subcellular transport and fate within the host cell? How are the multiple pathways for the T-DNA integration into the host cell genome regulated? Besides plant genetic transformation by *Agrobacterium* spp., the question is whether other natural cases of DNA transfer, via a similar mechanism, from different bacterial species to their eukaryotic hosts exist or have existed in past evolutionary times, and whether such events may have contributed to the gene flux from bacteria to eukaryotes. From a biotechnological viewpoint, better understanding of DNA transfer mechanisms will help expanding our toolbox for *Agrobacterium*-mediated transformation, for example, for improving the genetic transformation of "recalcitrant" plant species or of non-plant eukaryotic cells, or for a better control of the integration sites and integration patterns within the target genome. In this respect, the ultimate feat would be using synthetic biology to refactor the entire Ti plasmid (and, potentially, even the bacterial chromosome) to eliminate all pathogenic and transformation-unrelated (e.g., bacterial conjugation) abilities and to include non-bacterial (e.g., plant) genes known to facilitate transformation and/or transgene expression by refactoring them for optimal prokaryotic expression and export.

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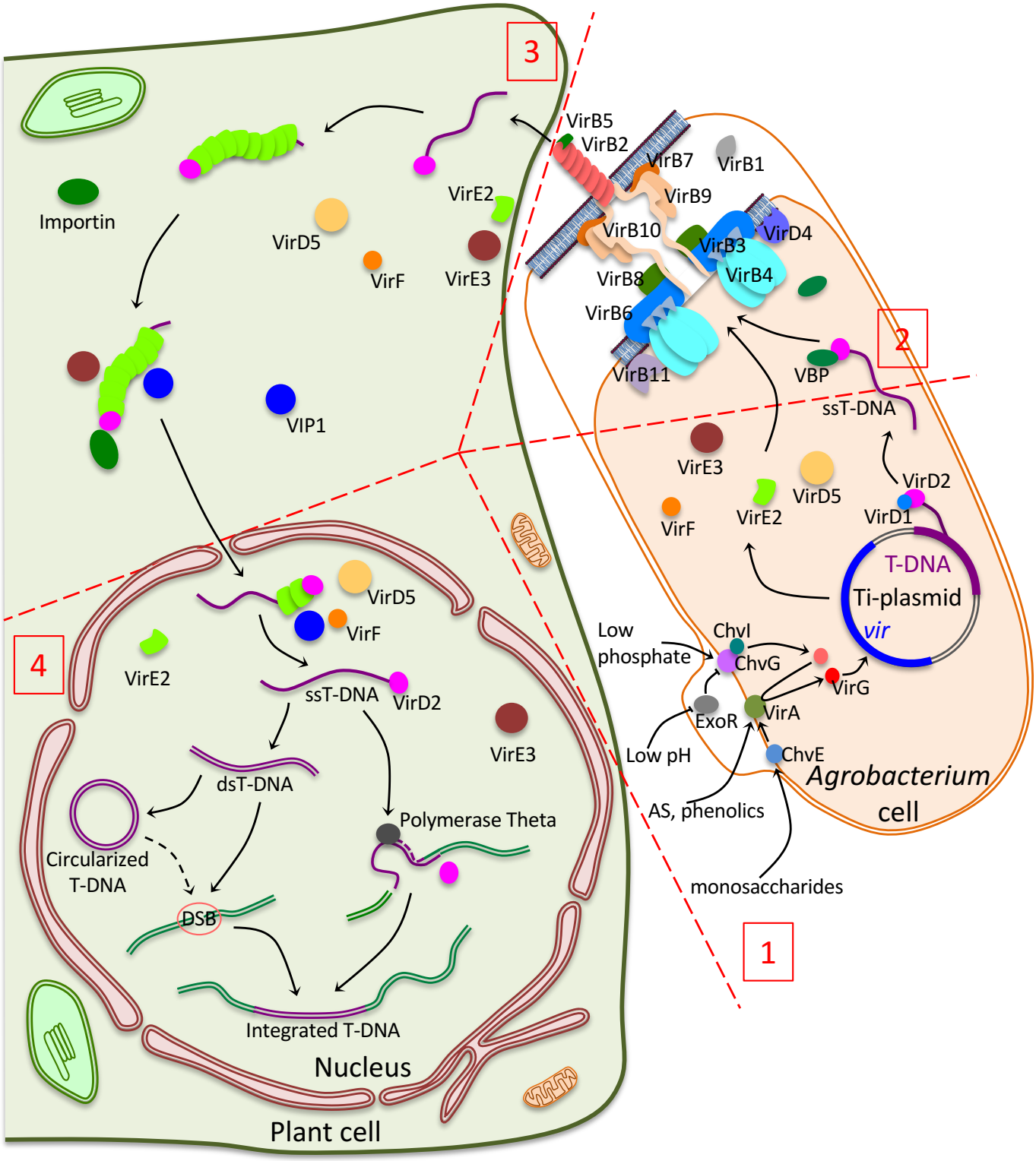
Abbreviations

AS: acetosyringone; DSB: double strand break; dsDNA: double-stranded DNA; HR: homologous recombination; IAA: indole acetic acid; LB: left border; MMEJ: microhomology-mediated end joining; NHEJ: non-homologous end joining; RB: right border; ssDNA: single-

stranded DNA; T-DNA: transferred DNA; T3SS: type three secretion system; T4SS: type four secretion system; Ti-plasmid: Tumor inducing plasmid; *vir*: virulence; UPP: unipolar polysaccharide; VBP: VirD2 binding protein; VIP1: VirE2 interacting protein 1; VIP2: VirE2 interacting protein 2.


3
VirB5
VirB2
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VirB3
VirD4
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VirB6
VirB11
VBP
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Importin
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VirD5
VirF
VirE3
VIP1
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VirD2
VirD1
VirF
T-DNA
Ti-plasmid
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VirD5
VirF
4
VirE2
ssT-DNA
VirD2
VirE3
Low
phosphate
ChvI
ChvG
VirG
ExoR
VirA
Low pH
ChvE
dsT-DNA
Polymerase Theta
Agrobacterium
cell
AS, phenolics
Circularized
T-DNA
DSB
monosaccharides
1
Integrated T-DNA
Nucleus
Plant cell

Legend to Figure

Fig. 1. Schematic representation of the main pathways of T-DNA transfer from *Agrobacterium* to the plant cell genome. See text for details.