Section 1
Agrobacterium-Mediated Transformation

1 Host Factors Involved in Genetic Transformation of Plant Cells by *Agrobacterium*

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Introduction

Agrobacterium tumefaciens and several other species of the *Agrobacterium* genus possess the unique ability to transfer a DNA segment from a specialized plasmid (tumor inducing or Ti plasmid in the case of *A. tumefaciens* and hairy root inducing or Ri plasmid for *Agrobacterium rhizogenes*, the two main species of pathogenic *Agrobacterium*) into a host plant cell. This feature is widely used in plant biotechnology, and *Agrobacterium* is, by far, the most important tool employed to produce transgenic plants (Newell 2000). Not surprisingly, the biology of *Agrobacterium* and its interactions with host plant have been the subject of numerous studies in the past three decades (for recent reviews, see Gelvin 2003; Citovsky *et al*. 2007; Dafny-Yelin *et al*. 2008).

In brief, the main steps of host genetic transformation mediated by *A. tumefaciens* are the following. The induction of *Agrobacterium*'s virulence machinery results in expression and activation of the virulence genes (*vir* genes) (Stachel *et al*. 1985b, 1986; McLean *et al*. 1994; Turk *et al*. 1994; Lee *et al*. 1996). This first step mobilizes a single-stranded DNA segment from the Ti or Ri plasmid. This segment of transferred DNA (T-DNA), delimited by two 25-bp direct repeat sequences known as left and right borders (LB and RB) (Peralta and Ream 1985; Wang *et al*. 1987), is termed the T-strand, and it represents the substrate of DNA transfer to the host cell. VirD2, associated with VirD1, forms a nuclease able to excise the T-strand by a strand-replacement mechanism, at the completion of which VirD2 remains covalently linked to the 5′-end (RB) of the T-strand (Ward and Barnes 1988; Young and Nester 1988; Durrenberger *et al*. 1989; Pansegrau *et al*. 1993; Jasper *et al*. 1994; Scheiffele *et al*. 1995; Relic *et al*. 1998). This VirD2–T-DNA complex is then translocated into the host cell cytoplasm by a mechanism relying on the VirB/VirD4 secretion system (Zupan *et al*. 1998; Vergunst *et al*. 2000; Christie 2004). The 11 proteins encoded by the VirB operon together with the VirD4 protein form a type IV secretion system, similar to the system allowing plasmid exchange by conjugation between bacteria. The type IV secretion system consists of a protein complex, spanning *Agrobacterium* internal membrane, periplasm and external membrane, and of an extracellular appendage, termed the T-pilus, composed mostly of VirB2 molecules forming a hollow channel (Christie *et al*. 2005). The VirB/VirD4 secretion system mediates the export of the VirD2–T-DNA complex out of the bacterial cytoplasm, and likely plays a role in its entry in the host cell. This secretion system is also required for the export of several *Agrobacterium* virulence proteins, that is, VirD5, VirE2, VirE3, and VirF, via their C-terminal secretion signals (Vergunst *et al*. 2000; Schrammeijer *et al*. 2003; Vergunst *et al*. 2003; 2005; Lacroix *et al*. 2005).

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There, the T-DNA–VirD2 complex is packaged by the single-stranded DNA-binding protein VirE2 (Christie *et al*. 1988; Citovsky *et al*. 1989; Sen *et al*. 1989). The resulting helical structure, called the T-complex, with the help of several bacterial and host proteins, is then imported into the host cell nucleus, targeted to the host chromatin, and ultimately integrated into the host genome (reviewed in Gelvin 2003; Lacroix *et al*. 2006a; Citovsky *et al*. 2007). The native T-DNA contains genes encoding enzymes that modify growth regulators and induce uncontrolled cell proliferation, which results in neoplastic cell growths (crown galls), and proteins mediating production and secretion of opines, amino acid, and sugar phosphate derivatives, secreted by the transformed cells and utilized almost exclusively by the *Agrobacterium* as carbon and nitrogen source (Escobar and Dandekar 2003).

The transfer of T-DNA is not sequence-specific, and any sequence of interest can be inserted between the T-DNA borders. The ability to engineer *Agrobacterium* to introduce genes of interest for plant genetic transformation is the basis of *Agrobacterium*'s use in biotechnology. The natural host range of *Agrobacterium* is very large, including most of the dicotyledonous and gymnosperm families (De Cleene and De Ley 1976). However, although the number of plant species transformable by *Agrobacterium* under laboratory conditions is always increasing (Newell 2000), in practice, producing transgenic plants efficiently is still a challenge for many plant species. Moreover, even nonplant species can be transformed by *Agrobacterium* under laboratory conditions (Lacroix *et al*. 2006b), including yeast (Bundock *et al*. 1995; Piers *et al*. 1996), various fungi (de Groot *et al*. 1998; Michielse *et al*. 2005), and cultured human cells (Kunik *et al*. 2001). This chapter focuses on numerous host plant factors that play important roles in the transformation process, from the initial interactions between *Agrobacterium* and plant cells and the activation of *Agrobacterium*'s virulence, to the integration of T-DNA into the host genome.

Plant Signals Affecting *Agrobacterium*'s Virulence Machinery

The rhizosphere is a complex and dynamic environment, where plant-associated bacteria such as *Agrobacterium* need subtle regulation systems to efficiently induce their virulence machinery (Brencic and Winans 2005). *Agrobacterium*'s virulence depends mostly on transcriptional activation of a set of virulence (*vir*) genes; this regulatory system allows the integration of environmental signals to ensure a timely expression of these genes. Moreover, the induction of virulence system obviously represents a high cost in energy for the bacterial cell, and its activation must be tightly regulated to ensure that it occurs only at the proximity of a susceptible host tissue. To this end, *Agrobacterium* harbors sensors able to recognize signals emitted by its host plants, and to activate the virulence machinery in response to these signals. The induction of *vir* gene expression in *Agrobacterium* relies on a two-component regulatory system encoded by the *virA* and *virG* genes that respond, directly or indirectly, to different plant and environmental cues (Klee *et al*. 1983; Stachel and Nester 1986). *virA* and *virG* have low basal expression, but their expression is highly inducible by a self-regulated system (Winans *et al*. 1988). The expression of other *vir* genes is virtually nonexistent in absence of induction, and it is strongly enhanced when the VirA–VirG system is activated. VirA–VirG represents a two-component regulatory system, in which VirA is the membrane-spanning sensor kinase that responds to external signals and activates the response regulator VirG by phosphorylation. Phosphorylated VirG recognizes and binds to a 12-bp long specific sequence, the *vir* box, which is present in all *vir* gene promoters, and serves to activate transcription (Brencic and Winans 2005).

Table 1.1. Plant and environmental signals that influence *Agrobacterium* virulence.

Phenotype	Signal	Bacterial receptors	References
Virulence activation	Phenolics (i.e., acetosyringone and related molecules)	VirA	Stachel *et al*. (1985a) and Lee *et al*. (1992, 1995)
	Monosaccharides	ChvE	Cangelosi *et al*. (1990) and Shimoda *et al*. (1990)
	Low pH	ChvG/ChvI	Melchers *et al*. (1989b) and Gao and Lynn (2005)
Virulence inhibition	DIMBOA, MDIBOA	VirA	Sahi *et al*. (1990) and Zhang *et al*. (2000)
	IAA	VirA	Liu and Nester (2006)
	Salicylic acid	VirA	Yuan *et al*. (2007) and Anand *et al*. (2008)
	Ethylene	Unknown	Nonaka *et al*. (2008b)

Several signals, from both host plants and the environment, can modulate *vir* gene expression (Table 1.1); these include phenolic compounds, monosaccharides, low pH, and low phosphate (McCullen and Binns 2006). Among these signals, only phenolics are absolutely required for virulence induction, whereas the other signals render *Agrobacterium* cells more sensitive to phenolics and/or enhance virulence induction levels.

Phenolic Compounds Activating Agrobacterium's Virulence

Initially, during the analyses of plant cell exudates, a single phenolic compound, acetosyringone (3,5-dimethoxyacetophenone) was identified. It was present at elevated concentrations and able to induce *vir* gene expression even in the absence of the plant cells (Stachel *et al*. 1985a, 1986; Bolton *et al*. 1986). Since then, more than 80 related phenolics, including glycoside derivatives (Joubert *et al*. 2004), have been shown to act as *vir* inducers with variable efficiency (Melchers *et al*. 1989a; Palmer *et al*. 2004). These studies revealed that all *vir*-inducing molecules share common structural features that enable this family of chemicals to interact with bacterial receptors and to act as virulence inducers, suggesting that these molecules are recognized by a unique bacterial receptor (Lee *et al*. 1992). Whereas direct interaction between radioactively labeled acetosyringone and VirA has not been detected (Lee *et al*. 1992), genetic studies have demonstrated that phenolic inducers most likely interact directly with the linker domain of VirA, thereby activating VirA's kinase activity (Lee *et al*. 1995). Indeed, the specific range of phenolic compounds recognized by different *Agrobacterium* strains was dependent on the *virA* locus, and could be transferred from one strain to another via the transfer of *virA*.

Reducing Monosaccharides

Sugar monomers are involved in *vir* gene activation in two ways: by enhancing VirA–VirG system sensitivity to phenols and by elevating the saturating concentration of phenols for virulence activation (Cangelosi *et al*. 1990; Shimoda *et al*. 1990). In addition, the range of phenolics recognized by the *Agrobacterium vir* gene induction system increases when monosaccharides are present as they act as coinducers (Peng *et al*. 1998). Several monosaccharides, such as D-glucose and D-galactose, are coinducers (Ankenbauer and Nester 1990; Shimoda *et al*. 1990), which

share minimal structural features (i.e., the presence of a pyranose ring and acidic groups), also suggesting that they are recognized by a specific receptor. The virulence response to monosaccharides indeed relies on a chromosome-encoded factor, ChvE. This periplasmic sugar-binding protein is believed first to bind monosaccharides, then to interact with the periplasmic domain of VirA, and to enhance the VirA ability to activate *vir* gene expression (Cangelosi *et al*. 1990; Lee *et al*. 1992; Shimoda *et al*. 1993; Banta *et al*. 1994).

Low pH and Low Phosphate

Low pH (i.e., ∼5.7) enhances virulence activation, and this effect is mediated by VirA (Melchers *et al*. 1989b; Chang *et al*. 1996) as well as ChvE (Gao and Lynn 2005). Low pH and low concentration of phosphate (both are frequently observed in a variety of soils) activate the *virG* expression (Winans 1990), likely by inducing another two-component regulatory system—also required for *vir* gene induction—composed of ChvG and ChvI (Charles and Nester 1993).

Production of Virulence Inducers by Plant Tissues

The presence of the *vir* gene inducers mentioned above can be associated with some characteristics of the plant cell or tissues susceptible to *Agrobacterium* DNA transfer. It is well known that wounded sites of the plant tissue are particularly susceptible to *Agrobacterium* infection (Smith and Townsend 1907), and wounding of plant tissue is thus classically used in many *Agrobacterium*-mediated plant genetic transformation protocols. Consistently, wound repair is usually associated with low pH, high activity of the phenylpropanoid pathway, and presence of monosaccharides involved in cell wall modification and synthesis (Baron and Zambryski 1995), showing that the most vulnerable sites for infection are usually associated with the presence of virulence-inducing signals. Moreover, phenolic compounds are classically secreted by plant roots in the rhizosphere, along with sugars, organic acids, amino acids, and other secondary metabolites (Walker *et al*. 2003).

Wounding is not absolutely required for infection (Escudero *et al*. 1995; Brencic *et al*. 2005); thus, alternative pathways of *Agrobacterium* infection are possible. Indeed, acetosyringone was first isolated from intact tissues, such as root exudates, and plant cell culture (Stachel *et al*. 1985a, 1986); thus, intact plant cells may release sufficient amount of phenolic compounds for *vir* gene induction. In addition, several studies of the modification of plant gene expression in response to *Agrobacterium* contact and infection have shown that many enzymes of the phenolic metabolism, potentially involved in the production of acetosyringone and other phenolic inducers of *Agrobacterium* virulence, are induced on interaction with *Agrobacterium* (Ditt *et al*. 2001, 2006; Veena *et al*. 2003). Consistently, the phenolic metabolism is modified in response to *Agrobacterium* infection (Simoh *et al*. 2009). Interestingly, phenolic molecules are usually produced by plants as part of defense reaction, and are toxic for many bacterial pathogens; *Agrobacterium* likely has evolved resistance to these molecules and utilize them as signals for induction of virulence.

In addition to their most important role as *vir* gene inducers, phenolics and monosaccharides also trigger a chemotactic response in *Agrobacterium*, directing the bacterial cell to move toward a potential point of infection in the plant tissue. Chemotaxis of *Agrobacterium* cells toward several *vir* inducer phenolics is constitutive and does not require *vir* gene induction (Parke *et al*. 1987), but relies on a chromosome-encoded cluster of genes (Wright *et al*. 1998).

Plant-Produced Inhibitors of Bacterial Virulence

Several extracellular plant metabolites are able to inhibit *Agrobacterium vir* gene expression and might, together with virulence inducers, contribute to the variability of susceptibility to *Agrobacterium* between plant species and tissues.

Homogenates of corn seedlings have a strong inhibitory effect on both growth and acetosyringone-dependent virulence activation of *A. tumefaciens* (Sahi *et al*. 1990). The substance responsible for this inhibitory effect was identified as DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one). DIMBOA, like indole acetic acid (IAA) and other auxins, is derived from the tryptophan biosynthetic pathway (Melanson *et al*. 1997). A similar molecule, MDIBOA (2-hydroxy-4,7-dimethoxybenzoxazin-3-one), is present at high concentration (up to 98%) in corn seedling root exudates. MDIBOA is also a potent inhibitor of *Agrobacterium* virulence, but has limited effect on bacterial growth (Zhang *et al*. 2000).

The auxin IAA itself inactivates *vir* gene expression by competing with the inducing phenolic compound acetosyringone for interaction with VirA (Liu and Nester 2006). In natural conditions, IAA is produced at relatively high concentrations by crown galls that develop after transformation, and is likely to inhibit new transformation.

Salicylic acid (SA) is a phenolic compound commonly produced by plants in response to many types of abiotic or biotic stress, and it is the major signal molecule of the systemic acquired resistance (SAR) in plants (Loake and Grant 2007). SA acts as an inhibitor of *vir* expression; most likely, SA shuts down *virA* and *virG* by attenuating the VirA protein kinase activity (Yuan *et al*. 2007), which would result in inhibition of expression of all *vir* genes. *Arabidopsis* mutants deficient in SA accumulation are more sensitive to *Agrobacterium* infection, whereas mutants overproducing SA are relatively recalcitrant (Yuan *et al*. 2007). Similar effects of SA on *vir* gene expression were observed in *Nicotiana benthamiana*, using either mutant plants altered in SA metabolism or exogenous application of SA (Anand *et al*. 2008).

The plant gaseous growth regulator ethylene was also suggested to inhibit the virulence of *Agrobacterium*. Indeed, plants impaired in ethylene production are more sensitive to *Agrobacterium*, whereas plants overproducing ethylene are more resistant (Nonaka *et al*. 2008b). Consistently, expression in *Agrobacterium* of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that degrades ACC, the immediate precursor of ethylene in higher plants, enhances the efficiency of *Agrobacterium* infection (Nonaka *et al*. 2008a). However, although these data suggest that ethylene might inhibit *Agrobacterium* virulence, a direct effect of ethylene on the *vir* gene regulation system has not been conclusively demonstrated.

Cell-to-Cell Contact and Passage of T-DNA through Host Cell Barriers

A close cell-to-cell contact is necessary for the T-DNA transfer from *Agrobacterium* to its host cell. Indeed, *Agrobacterium* mutants impaired in their ability to attach to plant cell generally show a diminished virulence (Matthysse 1987). Putative plant and *Agrobacterium* proteins that mediate cellular recognition and attachment have been suggested; however, the actual nature of the factors involved remains elusive. By analogy with other plant-associated Rhizobiaceae, a two-step mechanism was proposed (Smit *et al*. 1992; Rodriguez-Navarro *et al*. 2007). First, a contact between *Agrobacterium* and plant cells is initiated by as yet unidentified bacterial and plant extracellular receptors; these cellular interactions are believed to be nonspecific and reversible. Second, the attachment is consolidated by cellulose fibrils synthesized by the bacterial cells (Matthysse *et al*. 1981; Matthysse 1983).

Initial Cellular Interactions: Is There a Plant Cell Surface Receptor for Agrobacterium?

Plant lectins (proteins that bind reversibly to mono- or oligosaccharides) could play a role in binding bacterial exopolysaccharides (Hirsch 1999), as they do in the case of other Rhizobiaceae. Indeed, *A. tumefaciens* mutants in *chvA*, *chvB*, and *exoC* (*pscA*) that encode enzymes involved in the synthesis of an exocellular cyclic glucan (cyclic 1,2-β-D-glucan) were deficient in virulence, likely because of impaired attachment to the plant cell (Cangelosi *et al*. 1989; de Iannino and Ugalde 1989). However, the specific plant receptors for recognition of exocellular glucan produced by *Agrobacterium* have not been identified so far.

Rhicadhesin, an extracellular protein initially isolated from *Rhizobium*, inhibits attachment of *Rhizobium* and *Agrobacterium* to the plant cell surface when added exogenously, likely by saturating a putative plant cell surface receptor (Smit *et al*. 1989). It was, thus, suggested that *Agrobacterium* also encodes a similar protein, which might be responsible for initial attachment to plant cells, in a Ca^{2+}-dependent manner. However, the gene encoding an *Agrobacterium* rhicadhesin-like protein has not been identified, even though complete genome sequences have already become available for three *Agrobacterium* strains (Goodner *et al*. 2001; Wood *et al*. 2001; Slater *et al*. 2009). Several putative plant rhicadhesin-like receptors have been identified (Wagner and Matthysse 1992; Swart *et al*. 1994), but their actual functionality in *Agrobacterium* virulence has not been demonstrated. Because exogenous human vitronectin as well as antibodies against vitronectin inhibited binding of *Agrobacterium* to carrot cells, it was suggested that a vitronectin-like protein on the plant cell surface may bind bacterial rhicadhesin and thereby act as a receptor for initial attachment of *Agrobacterium* to the plant cell. However, recent data (Clauce-Coupel *et al*. 2008) demonstrated that whereas a vitronectin-like protein is present in the cell wall of plant tissues susceptible to *Agrobacterium*, this protein is involved neither in *Agrobacterium* attachment nor in its virulence. Using a bioassay based on suppression of rhicadhesin activity, a pea cell wall glycoprotein, which shows similarity to germin-like proteins present in many plant species, was also proposed to be a rhicadhesin receptor. Nevertheless, its actual interaction with rhicadhesin and its role in *Agrobacterium* infection have not been demonstrated.

Another series of putative plant proteins potentially involved in *Agrobacterium* attachment was identified using *Arabidopsis* insertional mutants, disrupted in genes encoding cell wall proteins. In a genetic screen for *Arabidopsis* mutants resistant to *Agrobacterium* (*rat* mutants) (Nam *et al*. 1999), several mutant lines impaired in their ability to allow *Agrobacterium* attachment were discovered. For example, the *rat1* phenotype results from the absence of expression of AtAGP17 (Gaspar *et al*. 2004). *Agrobacterium* attachment seems to be reduced in the *rat1* mutant, but the effect of the mutation might also be the result of other pathways, such as signaling or carbon allocation. *rat4* is deficient in CSLA9, a homolog of cellulose synthase (Zhu *et al*. 2003), the activity of which could modify the properties of the plant cell surface and influence bacterial attachment.

From the bacterial side, extracellular proteins involved in virulence, such as the components of the type IV secretion system, VirB1*, VirB2, and VirB5, might play a role in initial attachment (Aly and Baron 2007; Backert *et al*. 2008). A search for potential plant interactors of these proteins could help understand these cellular interactions. However, it remains unknown whether VirB1*, VirB2, and/or VirB5 are required at the earlier infection step of cell–cell recognition and attachment, or they function only later, during the transfer of DNA and proteins into the host cell cytoplasm. So far, the only identified bacterial factors essential both for attachment and for virulence are *chv*A, *chv*B, and *exo*C, which are all involved in exocellular oligosaccharide production. The *vir* region seems not to be essential for attachment, whereas the *att* region,

located in the pAt linear chromosome and initially considered to be involved in attachment (Matthysse *et al*. 1996; Matthysse and McMahan 1998), is not required for DNA transfer to plants, but mostly for control of quorum sensing (Nair *et al*. 2003).

Consolidation of Agrobacterium Attachment to Plant Cells by Cellulose Fibril Synthesis

In a second stage, the *Agrobacterium*–host cell interaction is consolidated by the production of cellulose fibrils by the bacterial cell, ending in irreversible binding and formation of bacterial aggregates at the plant cell surface. The mutants of *Agrobacterium* disrupted in the *celABCDE* operon were unable to form cellulose and showed a weaker attachment to plant cells as compared with wild-type bacteria (Matthysse 1983; Robertson *et al*. 1988). However, tumorigenicity of these mutants was only slightly reduced, but not completely blocked (Matthysse and McMahan 1998). Thus, this second step of attachment might not be absolutely necessary for T-DNA transfer, but it might be required to allow bacterial cells to remain in the vicinity of the transformed tissue (galls) and to use opines produced by the tumors. There are no known plant factors involved in binding of the bacterial cellulose fibrils on the plant cell surface.

When considering attachment of *Agrobacterium* cells to the host cell surface, the formation of bacterial biofilm in which bacteria are embedded appears to be essential for *Agrobacterium* virulence (Matthysse *et al*. 2005), and more generally for the virulence of many pathogenic bacteria (Danhorn and Fuqua 2007). Consistent with the ability of *Agrobacterium* to infect many different unrelated hosts, including nonplant species, it is uncertain whether there exists any absolutely required specific receptor(s) on the surface of the host cell; indeed, none of the putative receptors described above have ever been substantiated. Biofilm formation, which relies on the production of exocellular glucans, for example, cyclic 1,2-β-D-glucan and cellulose, could then be sufficient for the *Agrobacterium*'s attachment and virulence. Structural and chemical properties of the host cell surface could influence the genesis of biofilms.

Translocation of T-DNA and Virulence Proteins across the Plant Cell Wall and Plasma Membrane

The T-DNA and virulence proteins are exported from *Agrobacterium* via its VirB/VirD4 type IV secretion system (Ding *et al*. 2003; Christie 2004; Christie *et al*. 2005). The molecular details of T-DNA interactions with proteins of the VirB/VirD4 secretion system during transport through the bacterial membranes and periplasm were studied by coimmunoprecipitation (Cascales and Christie 2004). This study identified contacts of a T-DNA substrate with several subunits of the VirB/VirD4 system, and, using mutants in different *vir* genes, suggested the transport pathway for T-DNA substrate. However, this study was performed in bacteria and, thus, it provides information only about the first step of the T-DNA transfer, that is its export out of bacterial cells. The second step of the transfer process, that is, the passage of the translocated macromolecules through the host cell wall and plasma membrane, and the mechanism by which the extracellular proteins of the type IV secretion system, mainly VirB2, VirB5, and VirB7, could be involved in this process remain largely uncharacterized. During this second step of the T-DNA transfer, the T-pilus could act as a hollow needle allowing the injection of these macromolecules directly from the bacterial to the plant cytoplasm (Kado 2000), similar to how protein transport is mediated by type III secretion systems. However, the role of the T-pilus is

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still debated, and it could also function mainly by mechanically perforating the host cell wall and plasma membrane and allowing entry of macromolecules via another pathway (Llosa *et al*. 2002). Indeed, T-DNA transfer can occur in absence of detectable levels of T-pilus biogenesis; for example, the inhibition of T-pilus formation by blocking polymerization of VirB2 monomers does not abolish substrate transfer through the VirB/VirD4 type IV secretion system channel (Zhou and Christie 1997; Sagulenko *et al*. 2001; Jakubowski *et al*. 2005). Whether the VirD2–T-DNA complex and the exported virulence proteins move through the T-pilus or not, it is possible that plant factors interacting with components of the T-pilus and located in the plant cell wall, plasma membrane, and/or cytoplasm, play a role in this mechanism.

In a search for these putative receptors, four *Arabidopsis* proteins interacting with the processed C-terminal VirB2—that does not contain the 42-amino acid signal peptide, cleaved before T-pilus biogenesis—were identified (Hwang and Gelvin 2004). Three related proteins of unknown function, termed BTI1, 2, and 3, and a membrane-associated GTPase, AtRAB8, were found. Inhibition of expression of these proteins in *Arabidopsis* conferred relative resistance to *Agrobacterium*, whereas overexpression of BTI1 induced a hypersensitive phenotype. Although it is not clear exactly at which step these proteins might play a role, for example, during the initial attachment of *Agrobacterium* to the plant cell surface or later during the entry of the T-DNA, or virulence proteins into the host cell cytoplasm, they represent good candidates for host cell receptors required in the early *Agrobacterium*–plant cell interaction and/or macromolecule translocation.

Another possible pathway for translocation of the T-strand–VirD2 complex was suggested by the ability of the VirE2 molecule to form membrane-spanning channels, which allow passage of negatively charged macromolecules, such as oligonucleotides, in artificial lipid bilayers (Dumas *et al*. 2001). If this VirE2 channel also forms in plant cell membranes during the *Agrobacterium*–plant interaction, it may allow passage of macromolecules. Furthermore, the cooperative binding of VirE2 to the T-strand molecule during formation of the T-complex in the host cell cytoplasm may actively pull this DNA molecule, for example, out of the VirB/VirD4 and/or VirE2 channels, without the need for external energy sources (Grange *et al*. 2008). Although these activities of VirE2 have not been demonstrated *in vivo* so far, they might also involve interactions with plant factors in the cell wall or plasma membrane.

Overall, the host factors involved in macromolecular transfer between the *Agrobacterium* cell and the host cell cytoplasm are perhaps the least well characterized among all host factors that participate in the infection process. It is noteworthy that *Agrobacterium* can transfer DNA and proteins to numerous nonplant species (Lacroix *et al*. 2006b), suggesting a general nature of its macromolecular transfer machinery. That can be explained either by ancestral factors involved in host–pathogen interactions, which are conserved among eukaryotic organisms, or by the *Agrobacterium*'s ability to transport its macromolecules into host cell cytoplasm via a host-independent pathway, such as the one that does not rely on a specific host cell receptor.

Roles of Plant Factors in Transcytoplasmic Transport and Nuclear Import of the T-Complex

Structure of the T-Complex

The movement of a large DNA molecule, such as a segment of DNA of the typical size of the nopaline-type T-DNA (∼20 kilobases), is limited in the environment of the cytoplasm of a eukaryotic cell. In the cytoplasm, DNA movement could be impaired by molecular crowding

and, more importantly, by electrostatic associations. This is because DNA molecules are densely charged polyanions that could interact with many cellular components (Verkman 2002). Thus, large segments of free DNA are unlikely to reach the cell nucleus by simple diffusion. Indeed, studies in mammalian cells have shown that diffusion of circular or linear plasmid DNA molecules is extremely slow in the cytoplasm, and is negatively correlated with molecule size (Leonetti *et al*. 1991; Lukacs *et al*. 2000). Moreover, the free T-strand would form a polymeric random coil in the absence of packaging proteins. Typically, a randomly coiled free single-stranded DNA corresponding to a 20-kilobase T-DNA would reach a diameter, that is, the geometric mean of its extended length and its persistence length (Briels 1986), of about 300 nm; molecules of this size are unable to move freely in the cytoplasm and are also much larger than the nuclear pore exclusion limit of about 25 nm (Dworetzky and Feldherr 1988; Forbes 1992). Furthermore, even much larger T-DNA molecules, of up to 150 kilobases, can be transferred into the cells of tobacco (Hamilton *et al*. 1996) and tomato (Frary and Hamilton 2001) and integrated in their genomes. Packaging into transferable forms more suited for transcytoplasmic traffic and nuclear import is obviously required for such large molecules. Consequently, the T-strand must undergo a specific spatial organization that relies on interactions with packaging proteins in order to travel to, and subsequently enter the host cell nucleus. Indeed, within the host cell, the T-strand is thought to exist in a form of a nucleoprotein complex, the T-complex (Citovsky *et al*. 1988, 1989; Gelvin 1998).

In the T-complex, two bacterial virulence proteins, VirD2 and VirE2, which are essential for *Agrobacterium* virulence (Stachel *et al*. 1985a), directly associate with the T-strand (Young and Nester 1988; Citovsky *et al*. 1989; Sen *et al*. 1989). The T-complex is formed in the host cell cytoplasm (Figure 1.1, step 1) after VirE2 and the T-strand with covalently attached VirD2 are translocated independently of each other from *Agrobacterium* to the host cell (Otten *et al*. 1984; Citovsky *et al*. 1992; Gelvin 1998; Vergunst *et al*. 2000). Structural analyses of artificially reconstituted T-complexes (Citovsky *et al*. 1997; Abu-Arish *et al*. 2004; Grange *et al*. 2008) indicated that its diameter is about 15 nm (Abu-Arish *et al*. 2004); this is larger than the 9 nm diffusion limit of the nuclear pore (reviewed in Forbes 1992). Overall, the size of the T-complex suggests that its transport through the host cell cytoplasm and subsequent import into the nucleus occur by active mechanisms.

Roles of Molecular Motors and the Cytoskeleton in the T-Complex Movement through the Host Cell Cytoplasm

Before nuclear import can begin, the T-complex has to be transported across the cytoplasm from its point of entry and assembly to the cell nucleus. By analogy to many DNA viruses, which depend on dynein motors and microtubule networks for their transport toward the host cell nucleus, the transcytoplasmic transport of the T-complex might also represent an active process. Two lines of evidence support this notion. A plant VirE2-interacting protein 1 (VIP1) (Tzfira *et al*. 2001), which participates in nuclear import and intranuclear transport of the T-complex (see below), was shown to interact with the dynein-like DLC3 protein of *Arabidopsis*, suggesting a role for molecular motors in the T-complex movement through the cytoplasm (Tzfira 2006). That this movement might involve cytoskeletal elements is suggested by the observations that active transport of artificial T-complexes in a cell-free system occurs along the microtubule network (Salman *et al*. 2005). To date, the mechanism of the T-complex movement toward the host cell nucleus remains relatively unexplored and in need of more experimentation.

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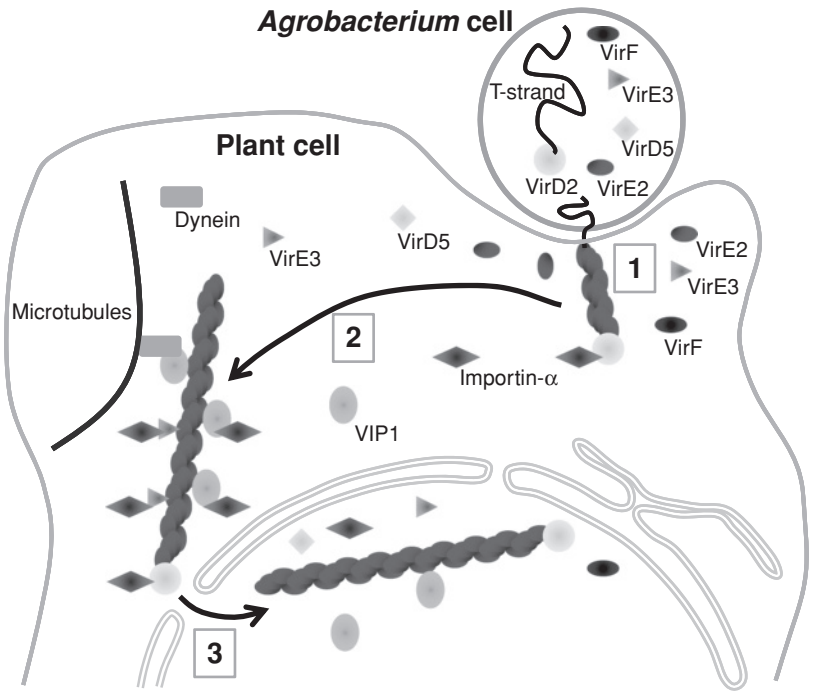

Figure 1.1. Nuclear import of the T-complex and virulence proteins. (1) The exported virulence proteins VirD5, VirE2, VirE3, and VirF and the T-strand covalently attached to VirD2 and enter independently of each other into the host cell cytoplasm via the VirB/VirD2 type IV secretion system. VirE2 then associates with the T-strand, forming the T-complex. Besides packaging the T-strand into a protected form suited for transcytoplasmic and nuclear transport, VirE2 might provide the energy needed for pulling the T-strand into the host cell. (2) Plant proteins interact with the T-complex and facilitate its movement across the cytoplasm toward the nuclear pore. The transcytoplasmic movement is likely mediated by the cytoskeleton and molecular motors; the latter might interact with VIP1, which is bound to VirE2. For nuclear targeting, VirD2 binds directly to importin-α, whereas VirE2 binds to VIP1 (and VirE3), which, in turn, binds to importin-α. (3) The T-complex passes through nuclear pores, likely in a polar manner, and importins are released inside the nucleus, whereas the T-complex is targeted to the host chromatin. The biological function of VirD5 in the process of the T-DNA transfer remains unknown. (For a color version of this figure, see Plate 1.)

Nuclear Import of the T-Complex

VirD2 is a nuclear protein when synthesized in eukaryotic cells, and it directly interacts with plant importin-α, which is a part of the cell nuclear import machinery that mediates the nuclear import of VirD2 (Ballas and Citovsky 1997). VirD2 carries two nuclear localization signals (NLSs), a monopartite N-terminal NLS and a bipartite C-terminal NLS (Herrera-Estrella *et al*. 1990; Howard *et al*. 1992; Tinland *et al*. 1992), but only the latter is essential for its nuclear import (Howard *et al*. 1992; Ziemienowicz *et al*. 2001). Several other plant VirD2 interactors could play a role in its subcellular localization. For example, *Arabidopsis* cyclophilins interact with VirD2 and might assist its nuclear targeting (Deng *et al*. 1998). In addition, VirD2 nuclear import might be regulated by phosphorylation/dephosphorylation of VirD2 itself. An enzymatically active type 2C serine/threonine protein phosphatase from tomato was found to interact with VirD2, and its overexpression resulted in inhibition of the VirD2 nuclear import (Tao *et al*. 2004).

Unlike VirD2, the nuclear import of VirE2 likely requires a more complex mechanism. VirE2 nuclear import in plant cells is strongly dependent on the presence of VIP1, a plant nuclear protein with a basic leucine zipper (bZIP) motif (Tzfira *et al*. 2001). VIP1, via its direct interactions with VirE2 and importin-α, likely links between VirE2 and the host nuclear import machinery (Tzfira *et al*. 2001, 2002). Consistently, *Agrobacterium*-mediated transformation efficiency is positively correlated with the expression level of VIP1 (Tzfira *et al*. 2001, 2002).

Interestingly, the VIP1's own nuclear import depends on its phosphorylation at a specific site (Djamei *et al*. 2007). This phosphorylation is mediated by the MAP kinase 3 (MPK3), an enzyme expressed as a part of a plant defense reaction that is elicited, among other factors, by *Agrobacterium*. MAP kinases are key factors in signal transduction during plant responses to many biotic and abiotic signals (Colcombet and Hirt 2008). It has been shown that an *Arabidopsis* insertional mutant in the *MPK3* gene is also resistant to *Agrobacterium* (Djamei *et al*. 2007). Thus, *Agrobacterium* might have evolved mechanisms to subvert the host defense response, that is, induction of MPK3 and phosphorylation of VIP1, to enhance its ability to infect its host (Djamei *et al*. 2007).

Recently, VirE2 has been shown to interact with some isoforms of plant importin-α, particularly importin-α-4 (Bhattacharjee *et al*. 2008); however, it is still unclear whether this interaction is functionally important for the VirE2 nuclear import. Generally, there might exist several pathways for nuclear import of VirE2 that *Agrobacterium* can utilize, depending on the host species and/or physiological conditions.

While it appears that there is redundancy between the roles of VirE2 and VirD2 in mediating T-DNA nuclear import, it is more likely that, in natural conditions, an efficient polar transport of the T-complex requires both factors (Figure 1.1, step 2) (Ziemienowicz *et al*. 2001). Both VirD2 (Ziemienowicz *et al*. 1999) and VirE2 (Zupan *et al*. 1996; Gelvin 1998) can mediate, independently of each other, nuclear import of short single-stranded DNA segments in animal (Ziemienowicz *et al*. 1999) and plant cells (Zupan *et al*. 1996; Gelvin 1998). The most likely mechanism, which is consistent with the polar structure of the T-complex, is that VirD2, attached to the 5′-end of the T-strand, directs the T-complex to the nuclear pore, while VirE2 and the associated VIP1, which presumably are distributed along the entire length of the T-strand, assist in its movement first through the cytoplasm (Tzfira 2006) and then through the nuclear pore (Ziemienowicz *et al*. 2001) (Figure 1.1, steps 2 and 3).

Another *Agrobacterium* virulence protein translocated to plant cells, VirE3, can interact with VirE2 and importin-α and facilitate the VirE2 nuclear import, thus partially mimicking the VIP1 function (Lacroix *et al*. 2005). Whereas VirE3 is not essential for plant genetic transformation, it is known to act as a host range factor of *Agrobacterium* (Hirooka and Kado 1986), potentially compensating for the lack or low amounts of VIP1-like proteins in some plant species. This strategy of *Agrobacterium* to improve its infection efficiency by exporting an effector protein that mimics functionally a host factor required for infection might represent a general adaptation of infectious microorganisms, including animal pathogens (Nagai and Roy 2003). Additionally, VirE3, as a nuclear protein in plant cells (Lacroix *et al*. 2005), could be involved in transcriptional regulation of yet unidentified host genes (Garcia-Rodriguez *et al*. 2006).

Remarkably, some strains of *A. rhizogenes* do not possess the *virE2* gene, yet are able to transfer and integrate DNA into their host genomes. In these strains, the function of VirE2 is likely fulfilled by the GALLS protein (Hodges *et al*. 2004, 2006, 2009) because virulence of an *A. tumefaciens* mutant in the *virE2* gene was restored by expression of the *A. rhizogenes GALLS* gene in the mutant bacterial cells (Hodges *et al*. 2004). Whether GALLS and VirE2 function

by the same molecular mechanism remains unclear. On the one hand, the full-length GALLS (Hodges *et al*. 2006) and VirE2 (Simone *et al*. 2001; Vergunst *et al*. 2003, 2005) both contain C-terminal signals for export from the bacterial cell through the type IV secretion system. Also, both GALLS (Hodges *et al*. 2006, 2009) and VirE2 (Citovsky *et al*. 1992, 1994; Tzfira and Citovsky 2001; Ziemienowicz *et al*. 2001) accumulate in the plant cell nucleus. Unlike VirE2, however, GALLS contains ATP-binding and helicase motifs (Hodges *et al*. 2006). The sequences of GALLS and VirE2 also do not share any homology.

Intranuclear Movement of the T-Complex and Its Uncoating

Chromatin Targeting of the T-Complex

Little is known about movement of the T-complex within the host nucleus toward the chromatin. Similarly to its transport in the cytoplasm, interactions of proteins coating the T-DNA with the host factors are likely to be involved (Figure 1.2, step 1).

When discussing chromatin targeting, it is important first to understand whether this targeting aims at specific sites within the genome or it is random. Several analyses of the T-DNA integration sites have shown that T-DNA integrates randomly into the host genome (Tinland

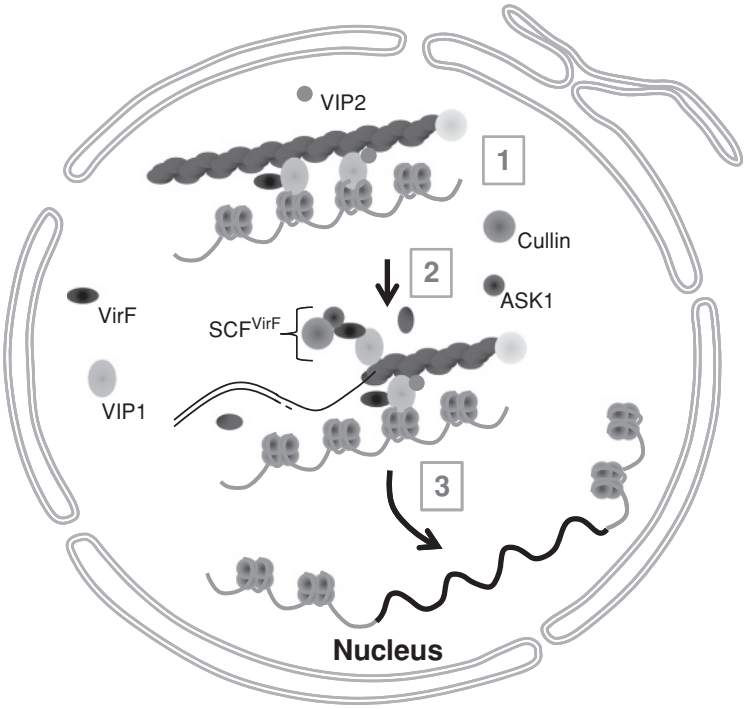

Figure 1.2. The fate of the T-complex in the host nucleus. (1) The T-complex is targeted to the host chromatin by a mechanism that might rely on the VIP1–nucleosome interaction and/or by interactions with proteins that target the DNA repair machinery to DSBs in the host genome. (2) Proteins associated with the T-DNA are removed by proteasomal degradation via the SCF^{VirF} pathway; at this stage, the T-strand is likely converted into a double-stranded form. (3) The T-DNA is integrated in the host genome by the host DNA repair machinery. (For a color version of this figure, see Plate 2.)

1996; Alonso and Stepanova 2003). Other studies suggested bias toward transcriptionally active chromatin and toward the regulatory regions of genes (Barakat *et al*. 2000; Chen *et al*. 2003; Schneeberger *et al*. 2005). However, this apparent bias might be an artifact of selection of high-expression transgenic plants. Recovery of transgenic plants in these studies relied on the expression of reporter or selectable marker genes that favored detection of integration events in transcriptionally active chromatin regions and caused underrepresentation of integration events in regions of low transcriptional activity. Indeed, two recent studies have shown that there is no integration bias when transgenic plants are recovered without selection that is dependent on expression of the transgene (Dominguez *et al*. 2002; Kim *et al*. 2007). Consequently, the *Agrobacterium* T-DNA most likely integrates randomly and, thus, has access to all areas of the host chromatin.

Several plant factors could assist the targeting of the T-complex to the host chromatin. CAK2M, a conserved plant ortholog of cyclin-dependent kinase-activating kinases, was identified as an interactor of VirD2 (Bako *et al*. 2003). CAK2M is a nuclear protein that also interacts with the largest subunit of RNA polymerase II, which recruits TATA box-binding proteins (TBPs). VirD2 was also found tightly associated with the TBP *in vivo* (Bako *et al*. 2003). Thus, VirD2 could play a role in the T-complex chromatin targeting by associating with CAK2M and/or TBP, which, in turn, naturally associate with the chromatin.

VIP1 is another candidate for a host factor involved in chromatin targeting of the T-complex. VIP1 is a transcription factor (Djamei *et al*. 2007), and as such it is expected to associate with the chromatin. Indeed, VIP1 was shown to bind to all four types of purified *Xenopus* core histones *in vitro* (Loyter *et al*. 2005), and to at least one plant core histone, H2A, *in vivo* (Li *et al*. 2005a). It was thus suggested that VIP1 acts as a molecular link between the VirE2 component of the T-complex and the core histone component of the host chromatin. This hypothesis is consistent with the known requirement of several core histones, and particularly H2A, for the T-DNA integration (Mysore *et al*. 2000b; Yi *et al*. 2002). Recent data indicate that VIP1 can have a strong interaction with purified plant nucleosomes *in vitro* that can be competitively inhibited by free histone H2A. In the same experiment, VIP1 also mediated binding of free VirE2 as well as a synthetic T-complex composed of VirE2 and single-stranded DNA to nucleosomes (Lacroix *et al*. 2008).

Because T-DNA integration occurs preferentially into double-stranded breaks (DSBs) in the host genome (Salomon and Puchta 1998; Chilton and Que 2003; Tzfira *et al*. 2003) (see also below), the proteins that recognize and target the DNA repair machinery to DSBs might also assist the targeting of the T-complex. Furthermore, another plant protein, VIP2, interacting with VirE2, was found necessary for stable plant transformation, but not for transient T-DNA gene expression (Anand *et al*. 2007). Thus, VIP2 presumably is involved in the T-complex chromatin targeting and/or T-DNA integration, although the mechanism of this involvement is not yet understood. VIP2 is a transcriptional regulator that modifies the expression levels of many genes, including the core histones (Anand *et al*. 2007), and its effect on the *Agrobacterium* T-DNA integration may also be indirect, via altering the expression of histones.

Proteasomal Uncoating of the T-Complex

The removal ("uncoating") of the proteins protecting the T-strand is necessary to allow the second strand synthesis, which likely occurs before integration (Chilton and Que 2003; Tzfira *et al*. 2003) (Figure 1.2, step 2) as well as to expose the T-DNA to the host cell DNA repair machinery that mediates the integration event (Tzfira *et al*. 2004a).

The first indication that proteasomal degradation may be involved in the uncoating process was provided by the presence of an F-box domain in VirF (Regensburg-Tuink and Hooykaas 1993), an *Agrobacterium* virulence protein exported to the host plant (Vergunst *et al*. 2000). In eukaryotic cells, F-box proteins represent a component of the Skp1/Cullin/F-box protein (SCF) E3 ligase complex, and they function to recognize and direct specific substrates to degradation by the 26S proteasome (reviewed in Deshaies 1999; Kipreos and Pagano 2000; Cardozo and Pagano 2004). VirF interacts with *Arabidopsis* Skp1-like protein 1 (ASK1), a plant homolog of the yeast Skp1 protein (Schrammeijer *et al*. 2001; Tzfira *et al*. 2004b), and both VirF and ASK1 localize in the plant cell nucleus (Tzfira *et al*. 2004b), where the T-complex uncoating is expected to occur. One of the cellular substrates recognized by VirF is VIP1; VirF binds VIP1 and destabilizes it in plants and in yeast cells (Tzfira *et al*. 2004b), which are known to be genetically transformed by *Agrobacterium* (Bundock *et al*. 1995; Piers *et al*. 1996). In addition, VirF, which does not bind VirE2, promotes VirE2 destabilization in the presence of VIP1 (Tzfira *et al*. 2004b), suggesting that VirF can destabilize the entire VIP1–VirE2 complex. In yeast, VIP1 and VirE2 destabilization by VirF is Skp1-dependent as it does not occur in an *skp1-4* mutant (Connelly and Hieter 1996), indicating that this destabilization occurs via the SCF^{VirF} pathway (Tzfira *et al*. 2004b). That VirF might help to uncoat the T-complex, docked at the host chromatin, is supported by the ability of VirF to associate simultaneously with purified VIP1, VirE2, single-stranded DNA, and nucleosomes *in vitro* (Lacroix *et al*. 2008). The involvement of the 26S proteasome in *Agrobacterium* infection is consistent with the inhibitory effect of the proteasomal inhibitor MG132 on the transformation process (Tzfira *et al*. 2004b).

Historically, VirF has been considered to be a bacterial host range factor (Melchers *et al*. 1990; Regensburg-Tuink and Hooykaas 1993). For example, VirF enhances *Agrobacterium* infectivity in tomato and *Nicotiana glauca* (Regensburg-Tuink and Hooykaas 1993), but it is not required for infection of tobacco or *Arabidopsis*. Thus, in plant species for which infection does not require VirF, the plant might produce proteins that have F-box protein functions that can substitute for VirF during transformation. Among several *Arabidopsis* F-box proteins induced by *Agrobacterium* infection (Ditt *et al*. 2006), we have identified one, designated VIP1-binding F-box protein (VBF), that binds VIP1 and promotes proteasomal destabilization of VIP1 and VIP1–VirE2 complexes in yeast and plants. Moreover, suppression of VBF expression in *Arabidopsis* reduced their susceptibility to *Agrobacterium*-induced tumor formation (Adi Zaltsman and Vitaly Citovsky, unpublished data).

T-DNA Integration into the Host Genome

Recent advances have substantially enhanced our understanding of the T-DNA integration pathways and uncovered many host factors that participate in these events (Tzfira *et al*. 2004a). The likely model for *Agrobacterium* T-DNA integration includes two major steps: first, the T-strand is converted to a double-stranded form; second, the host cell DNA repair machinery mediates the double-stranded T-DNA integration into DSBs in the host genome (Tzfira *et al*. 2004a).

Early studies of the T-DNA integration mechanisms focused on the role of virulence proteins accompanying the T-DNA. In particular, VirD2 was suggested to act as an integrase or a ligase because it contains an H-R-Y motif typical of the phage λ integrase (Tinland *et al*. 1995), and it can cleave and ligate single-stranded DNA *in vitro* (Pansegrau *et al*. 1993). However, mutations in the H-R-Y motif reduce precision of T-DNA integration, but not its efficiency (Tinland *et al*. 1995), and the cleavage/ligation activity was strictly sequence-specific (Pansegrau *et al*. 1993),

which is not consistent with the direct function of VirD2 in integration. A later study revealed that, in fact, VirD2 itself does not act as a DNA ligase *in vitro*, suggesting that T-DNA integration is more likely to be mediated by host enzymes (Ziemienowicz *et al*. 2000). It cannot be ruled out, however, that VirD2 is involved in T-DNA integration by recruiting host plant factors that mediate integration.

The first proposed T-DNA integration model (Tinland 1996), named SSGR (single-stranded gap repair), was based on the sequence of a few T-DNA integration sites. In this model, T-DNA integration started by annealing of the T-DNA right border to microhomologies in the host genomic DNA, followed by synthesis of the second strand and ligation of the left border (Tinland 1996). This model was challenged by subsequent data (Tzfira *et al*. 2004a). On the one hand, several T-DNA integration patterns, incompatible with the SSGR model, have been discovered. Analysis of a large number of T-DNA integration sites in plant genomes revealed that microhomologies are not consistently observed at these sites (Alonso *et al*. 2003). Moreover, some complex integration patterns involving multiple T-DNAs, which can be integrated at the same site in direct or reverse orientation and with or without filler DNA, cannot be explained by the SSGR model (De Neve *et al*. 1997; De Buck *et al*. 1999). Specifically, the occurrence of two T-DNA molecules integrated in a head-to-head orientation is not compatible with the SSGR model because head-to-head recombination is not possible for single-stranded DNA. In addition, the presence of filler DNA cannot be explained by the SSGR model. On the other hand, increasing evidence points to a role for DSBs and the DSB repair machinery in T-DNA integration, suggesting that T-strands are converted to double-strand molecules before integration. Generation of DSBs in the plant genome by a rare-cutting DNA endonuclease resulted in higher frequencies of foreign DNA integration, in direct transformation (Salomon and Puchta 1998) as well as in *Agrobacterium*-mediated transformation (Salomon and Puchta 1998). Finally, the use of rare-cutting endonuclease sites present both in the host DNA and in the T-DNA provided direct proof that T-strand becomes double-stranded before integration (Chilton and Que 2003; Tzfira *et al*. 2003). In these studies, integration of T-DNA molecules digested *in vivo* by the rare-cutting endonuclease was observed, including precise ligation of the T-DNA, which reconstituted the original enzyme recognition site at the junction between the T-DNA and the host DNA. Because the endonuclease used in these studies can cleave only double-stranded DNA, the T-strand must have been converted into the double-stranded form prior to integration. Overall, T-DNA integration occurs preferentially at DSBs, as shown by analysis of frequency of integration (Salomon and Puchta 1998; Chilton and Que 2003; Tzfira *et al*. 2003; Windels *et al*. 2003). This observation suggests that DSBs "attract" T-DNA for integration, and it is consistent with the higher frequency of transgene integration after X-ray treatment, known to induce DSBs (Leskov *et al*. 2001).

The use of *Saccharomyces cerevisiae* as a heterologous host for *Agrobacterium* (Bundock *et al*. 1995) has been instrumental for the identification of host factors mediating the T-DNA integration. These experiments strongly indicated that T-DNA integration depends mostly on host factors. Indeed, whereas in plants, the T-DNA integration occurs mostly by nonhomologous recombination (NHR), integration by homologous recombination (HR) can occur in yeast, provided that the T-DNA contains sequence homology to a target sequence in the yeast genome. Using yeast mutants in HR or NHR machinery made it possible to direct the T-DNA integration toward the remaining pathway. From the two key enzymes involved in these integration mechanisms, Rad52, a single-stranded DNA-binding protein was necessary for HR (van Attikum *et al*. 2001), and Ku70, a double-stranded DNA-binding protein that functions in a heterodimer with Ku80, was required for NHR (van Attikum and Hooykaas 2003). No T-DNA integration at all was observed in mutants in both of these genes. The involvement of

other cellular proteins in each of these T-DNA integration pathways was also demonstrated (van Attikum *et al*. 2001; van Attikum and Hooykaas 2003). For example, Rad51 is involved in homologous DNA pairing and strand exchange reaction (Sung *et al*. 2003), and during yeast transformation by *Agrobacterium*, Rad51 was required for T-DNA integration via the HR pathway (van Attikum and Hooykaas 2003). Interestingly, an *Arabidopsis* mutant in the *RAD5* gene, which is closely related to the yeast *RAD51*, displayed a reduced susceptibility to *Agrobacterium* (Sonti *et al*. 1995). The Mre11 protein functions in complex with Rad50 and Xrs2, and it has an exonuclease activity that plays a role in both HR and NHR (Usui *et al*. 1998). In *Agrobacterium*-infected yeast cells, Mre11 was necessary for T-DNA integration via the NHR pathway (van Attikum *et al*. 2001). Taken together, these observations strongly suggest that, at least in yeast, T-DNA integration is mainly dependent on the host, rather than bacterial factors.

In higher plants, integration of foreign DNA occurs mainly by NHR, although HR can also take place, albeit at extremely low rates (Gheysen *et al*. 1991; Mayerhofer *et al*. 1991; Terada *et al*. 2002). Unlike yeast mutants, experiments with *Arabidopsis* mutants in the HR and NHR pathways were difficult to interpret. For example, the *Arabidopsis* ligase AtLig4 (Friesner and Britt 2003) and AtKu80 were reported to be required (Friesner and Britt 2003; Li *et al*. 2005b) or dispensable (Gallego *et al*. 2003) for T-DNA integration. These discrepancies and differences from the yeast system might reflect more complex and redundant pathways for HR and NHR in plants, as well as the differences between the techniques, that is, floral-dipping versus root tissue regeneration, used for transformation; interestingly, floral-dipping that transforms germline cells is effective even in mutants that are resistant to transformation of roots (Mysore *et al*. 2000a). The T-DNA integration pathways in plants can be modified by expression of yeast components of the HR pathway. Specifically, expression of the yeast *RAD54* in transgenic *Arabidopsis* leads to a two-order-of-magnitude increase in the frequency of T-DNA integration by HR (Shaked *et al*. 2005). *RAD54* is a member of the *SWI2/SNF2* superfamily of chromatin remodeling genes known to promote recombination between homologous DNA segments in yeast (Tan *et al*. 2003); moreover, *RAD54* disruption leads to lower rates of targeted gene integration in yeast and in animal cells (Bezzubova *et al*. 1997; Essers *et al*. 1997).

In addition to the DNA repair machinery, plant host factors are important for T-DNA integration. For example, core histones, such as H2A, are required for an efficient T-DNA integration into the host genome (Mysore *et al*. 2000b; Yi *et al*. 2002). The role of core histones in T-DNA integration can, in turn, be related to the ability of VIP1 to link between them and the T-complex (see above). Chromatin assembly factor 1 (CAF), which is involved in chromatin remodeling, might represent a plant factor that negatively regulates T-DNA integration, potentially by modifying the target chromatin structure. *Arabidopsis* plants deficient in CAF were more sensitive to stable transformation by *Agrobacterium* (Endo *et al*. 2006). Interestingly, deficiency in CAF also increased the frequency of T-DNA integration by HR (Endo *et al*. 2006). Finally, VIP2 is also known to be required for the T-DNA integration (Anand *et al*. 2007).

Our present knowledge about T-DNA integration is synthesized into the model, shown in Figure 1.3 (Tzfira *et al*. 2004a). In this model, the T-strand is first converted to double-stranded DNA, likely by host cell factors (i.e., the host DNA replication machinery) that have yet to be characterized. Then, the double-stranded T-DNA is targeted to DSBs in the host genome, potentially via interaction with Ku70 and Ku80 as well as other proteins involved in DSB repair. At this stage, it is possible that several T-DNA molecules become ligated to each other, resulting in multiple T-DNA integrations in different orientations and patterns. The T-DNA or T-DNA multimers are then ligated to the free ends of the DSB by the components of the host DSB repair machinery.

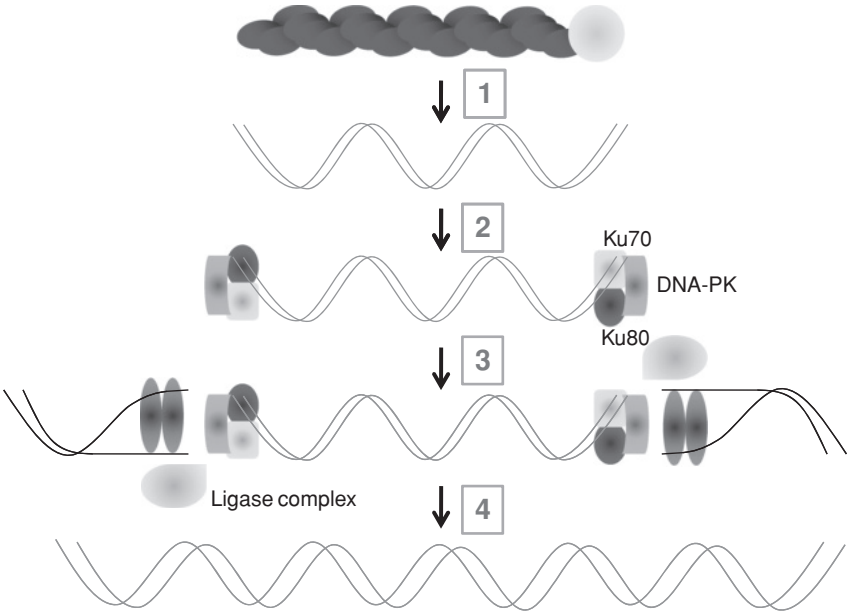

Figure 1.3. Integration of T-DNA into the host genome. (1) The T-DNA is uncoated of its associated proteins and converted to a double-stranded molecule. (2) Proteins of the DSB repair machinery interact with the double-stranded T-DNA molecule, and might assist its targeting to DSBs in the host genome. (3) The T-DNA associates with a DSB site in the host genome. (4) Host proteins mediate the ligation of the T-DNA into DSB. (For a color version of this figure, see Plate 3.)

Activation and Modulation of the Host Plant Defense Reaction

Agrobacterium infection does not elicit hypersensitive response or tissue necrosis in the host. However, on the basis of genomic and proteomic analyses, *Agrobacterium* cellular contact with infection of the host alters expression of numerous host defense response genes.

Two studies in cultured plant cells have shown that many genes involved in plant defense are induced by inoculation with *Agrobacterium* (Ditt *et al*. 2005, 2006). Similarly, the expression of several defense response genes, such as those encoding β-1,3-glucanase and phenylalanine ammonia-lyase, is activated during *Arabidopsis* infection by *Agrobacterium* (Veena *et al*. 2003). Importantly, this work suggests that plant response to *Agrobacterium* takes place in two distinct stages. First, during the initial 12 hours after inoculation, a "general" response is observed with activation of many defense-related genes, which is very similar to the general plant response to biotic stress. Second, after these 12 hours, most of the defense-related genes are downregulated to their initial levels, and a second set of host genes involved in cell division and cell growth, which might be important for the transformation process, is activated (Veena *et al*. 2003). Thus, there must be a mechanism by which, during more advanced stages of the infection, *Agrobacterium* is able to repress expression of plant defense-related genes, avoiding the initiation of hypersensitive response in the infected tissue. The nature of this mechanism remains unknown.

RNA silencing is a common host defense reaction against many plant viruses, while viruses have developed various antisilencing strategies to suppress this defense (Ding and Voinnet 2007). Similarly, during *Agrobacterium* infection, small interfering RNAs (siRNAs), which often mediate RNA silencing (Ding and Voinnet 2007), specific for the T-DNA sequences are

produced by the host plant (Dunoyer *et al*. 2006). Thus, plants respond to the invading bacterial and viral genetic material in a similar fashion. This RNA silencing-based innate immunity underlies the relatively short, 2–3 days after *Agrobacterium* inoculation, period during which the T-DNA is transiently expressed at high levels in most transformation experiments. Most likely, after this period, the RNA silencing response takes effect, reducing or even eliminating T-DNA expression. Indeed, coexpression with plant viral silencing suppressors, such as P19 or HCPro, substantially enhances and prolongs transient T-DNA expression (Voinnet *et al*. 2003).

It is tempting to speculate that, similarly to plant viruses that encode RNA silencing suppressors that overcome the host defense (Li and Ding 2001; Wang and Metzlaff 2005; Levy *et al*. 2008), *Agrobacterium* also has evolved a mechanism to export a suppressor into the host cell, either as a protein effector or as a T-DNA-encoded gene. To date, no such *Agrobacterium* factor has been identified. Recent data indicate that a decrease in siRNAs specific for the oncogenic T-DNA is observed within tumors (Dunoyer *et al*. 2006). This antisilencing effect, however, appeared to be an indirect consequence of modification of the hormonal status of the tumor tissue rather than the activity of a putative *Agrobacterium* silencing suppressor (Dunoyer *et al*. 2006).

Interestingly, *Agrobacterium* infection also suppresses the SAR response of the host plant. SA accumulation, as well as the expression levels of pathogenesis-related genes *PR-1* and *PR-5*, was lower in *Agrobacterium*-infected *Arabidopsis* plants than in healthy plants (Gaspar *et al*. 2004). Modulation of SAR by *Agrobacterium* might involve AtAGP17, an *Arabidopsis* lysine-rich arabinogalactan protein; a mutant in AtAGP17 (*rat1*) was resistant to *Agrobacterium* and did not exhibit reduced expression of *PR-1* and *PR-5* on *Agrobacterium* infection (Gaspar *et al*. 2004). However, the effect of *Agrobacterium* infection on the *PR* gene expression might vary by host species. In tobacco, the *PR-1* gene expression is, in fact, induced following *Agrobacterium* inoculation (Pruss *et al*. 2008). The elevated level of PR-1 was sufficient to elicit resistance to *Tobacco mosaic virus* (TMV). This induction of the host defense by *Agrobacterium* did not depend on the presence of T-DNA, and it was reduced only slightly when the entire Ti plasmid was absent. Thus, *PR-1* induction likely represents a general host response to bacterial challenge rather than a specific reaction to transfer of foreign genetic material and/or proteins (Pruss *et al*. 2008). *Agrobacterium* infection of tobacco plants also elevated the levels of miR393, a microRNA that represses auxin signaling and contributes to antibacterial resistance. Unlike *PR-1* induction, the miR393 induction depended on the presence of the oncogenic Ti plasmid (Pruss *et al*. 2008).

An additional plant defense pathway that could target *Agrobacterium* involves the host cell caspase-like proteases. These enzymes, involved in the programmed cell death in plants and induced in tobacco by TMV infection, have been shown to cleave specifically the VirD2 protein (Chichkova *et al*. 2004). It remains unclear whether *Agrobacterium* infection itself can induce the caspase-based response. However, once induced, this response appears to defend against the *Agrobacterium*-mediated transformation, because mutating VirD2 to render it insensitive to caspase cleavage enhanced transformation efficiency (Reavy *et al*. 2007). Overall, the *Agrobacterium*–plant cell interaction appears to induce, as well as suppress, a variety of host defense mechanisms, and the end result of these reactions and counterreactions might contribute to the differences among plant species' susceptibility to *Agrobacterium*.

Concluding Remarks

As more and more host factors involved in *Agrobacterium* infection are discovered, the complexity of the mechanism by which T-DNA is transported into the host plant cell and incorporated

into its genome is revealed. That *Agrobacterium* can transfer DNA into many kinds of organisms, plants and nonplants, indicates either that the mechanisms involved rely on widespread evolutionary-conserved host factors, or that the *Agrobacterium* DNA transfer machinery has been able to adapt itself to various host species and utilize different pathways, depending on the host. The value of increasing our knowledge about these host factors is twofold. First, it allows us to better understand the basic cellular biology processes and systems among eukaryotes involved in diverse and fundamental processes such as cell–cell recognition, macromolecular transport across membranes, nuclear and intranuclear transport, chromatin targeting, and DNA repair and integration. Second, it opens new possibilities to improve the transformation efficiency of the many plant species that remain difficult for both research and biotechnology applications.

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References

Abu-Arish A, Frenkiel-Krispin D, Fricke T, Tzfira T, Citovsky V, Wolf SG, Elbaum M (2004) Three-dimensional reconstruction of *Agrobacterium* VirE2 protein with single-stranded DNA. *Journal of Biological Chemistry* **279**, 25359–25363.

Alonso JM, Stepanova AN (2003) T-DNA mutagenesis in *Arabidopsis*. *Methods in Molecular Biology* **236**, 177–188.

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**, 653–657.

Aly KA, Baron C (2007) The VirB5 protein localizes to the T-pilus tips in *Agrobacterium tumefaciens*. *Microbiology* **153**, 3766–3775.

Anand A, Krichevsky A, Schornack S, Lahaye T, Tzfira T, Tang Y, Citovsky V, Mysore KS (2007) *Arabidopsis* VIRE2 INTERACTING PROTEIN2 is required for *Agrobacterium* T-DNA integration in plants. *Plant Cell* **19**, 1695–1708.

Anand A, Uppalapati SR, Ryu CM, Allen SN, Kang L, Tang Y, Mysore KS (2008) Salicylic acid and systemic acquired resistance play a role in attenuating crown gall disease caused by *Agrobacterium tumefaciens*. *Plant Physiology* **146**, 703–715.

Ankenbauer RG, Nester EW (1990) Sugar-mediated induction of *Agrobacterium tumefaciens* virulence genes: structural specificity and activities of monosaccharides. *Journal of Bacteriology* **172**, 6442–6446.

Backert S, Fronzes R, Waksman G (2008) VirB2 and VirB5 proteins: specialized adhesins in bacterial type-IV secretion systems? *Trends in Microbiology* **16**, 409–413.

Bako L, Umeda M, Tiburcio AF, Schell J, Koncz C (2003) The VirD2 pilot protein of *Agrobacterium*-transferred DNA interacts with the TATA box-binding protein and a nuclear protein kinase in plants. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 10108–10113.

Ballas N, Citovsky V (1997) Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 10723–10728.

Banta LM, Joerger RD, Howitz VR, Campbell AM, Binns AN (1994) Glu-255 outside the predicted ChvE binding site in VirA is crucial for sugar enhancement of acetosyringone perception by *Agrobacterium tumefaciens*. *Journal of Bacteriology* **176**, 3242–3249.

Barakat A, Gallois P, Raynal M, Mestre-Ortega D, Sallaud C, Guiderdoni E, Delseny M, Bernardi G (2000) The distribution of T-DNA in the genomes of transgenic *Arabidopsis* and rice. *FEBS Letters* **471**, 161–164.

Baron C, Zambryski PC (1995) The plant response in pathogenesis, symbiosis, and wounding: variations on a common theme? *Annual Review of Genetics* **29**, 107–129.

Bezzubova O, Silbergleit A, Yamaguchi-Iwai Y, Takeda S, Buerstedde JM (1997) Reduced X-ray resistance and homologous recombination frequencies in a RAD54-/- mutant of the chicken DT40 cell line. *Cell* **89**, 185–193.

Bhattacharjee S, Lee LY, Oltmanns H, Cao H, Veena, Cuperus J, Gelvin SB (2008) IMPa-4, an *Arabidopsis* importin alpha isoform, is preferentially involved in *Agrobacterium*-mediated plant transformation. *Plant Cell* **20**, 2661–2680.

Bolton GW, Nester EW, Gordon MP (1986) Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. *Science* **232**, 983–985.

Brencic A, Winans SC (2005) Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. *Microbiology and Molecular Biology Reviews* **69**, 155–194.

Brencic A, Angert ER, Winans SC (2005) Unwounded plants elicit *Agrobacterium* vir gene induction and T-DNA transfer: transformed plant cells produce opines yet are tumour free. *Molecular Microbiology* **57**, 1522–1531.

Briels W (1986) *The Theory of Polymer Dynamics*. Clarendon Press, Oxford.

Bundock P, den Dulk-Ras A, Beijersbergen A, Hooykaas PJ (1995) Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *EMBO Journal* **14**, 3206–3214.

Cangelosi GA, Martinetti G, Leigh JA, Lee CC, Theines C, Nester EW (1989) Role for *Agrobacterium tumefaciens* ChvA protein in export of beta-1,2-glucan. *Journal of Bacteriology* **171**, 1609–1615.

Cangelosi GA, Ankenbauer RG, Nester EW (1990) Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 6708–6712.

Cardozo T, Pagano M (2004) The SCF ubiquitin ligase: insights into a molecular machine. *Nature Reviews Molecular Cell Biology* **5**, 739–751.

Cascales E, Christie PJ (2004) Definition of a bacterial type IV secretion pathway for a DNA substrate. *Science* **304**, 1170–1173.

Chang CH, Zhu J, Winans SC (1996) Pleiotropic phenotypes caused by genetic ablation of the receiver module of the *Agrobacterium tumefaciens* VirA protein. *Journal of Bacteriology* **178**, 4710–4716.

Charles TC, Nester EW (1993) A chromosomally encoded two-component sensory transduction system is required for virulence of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **175**, 6614–6625.

Chen S, Jin W, Wang M, Zhang F, Zhou J, Jia Q, Wu Y, Liu F, Wu P (2003) Distribution and characterization of over 1000T-DNA tags in rice genome. *Plant Journal* **36**, 105–113.

Chichkova NV, Kim SH, Titova ES, Kalkum M, Morozov VS, Rubtsov YP, Kalinina NO, Taliansky ME, Vartapetian AB (2004) A plant caspase-like protease activated during the hypersensitive response. *Plant Cell* **16**, 157–171.

Chilton MD, Que Q (2003) Targeted integration of T-DNA into the tobacco genome at double-stranded breaks: new insights on the mechanism of T-DNA integration. *Plant Physiology* **133**, 956–965.

Christie PJ (2004) Type IV secretion: the *Agrobacterium* VirB/D4 and related conjugation systems. *Biochimica Et Biophysica Acta* **1694**, 219–234.

Christie PJ, Ward JE, Winans SC, Nester EW (1988) The *Agrobacterium tumefaciens* virE2 gene product is a single-stranded-DNA-binding protein that associates with T-DNA. *Journal of Bacteriology* **170**, 2659–2667.

Christie PJ, Atmakuri K, Krishnamoorthy V, Jakubowski S, Cascales E (2005) Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annual Review of Microbiology* **59**, 451–485.

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

Citovsky V, Wong ML, Zambryski P (1989) Cooperative interaction of *Agrobacterium* VirE2 protein with single-stranded DNA: implications for the T-DNA transfer process. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 1193–1197.

Citovsky V, Zupan J, Warnick D, Zambryski P (1992) Nuclear localization of *Agrobacterium* VirE2 protein in plant cells. *Science* **256**, 1802–1805.

Citovsky V, Warnick D, Zambryski P (1994) Nuclear import of *Agrobacterium* VirD2 and VirE2 proteins in maize and tobacco. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 3210–3214.

Citovsky V, Guralnick B, Simon MN, Wall JS (1997) The molecular structure of *Agrobacterium* VirE2-single stranded DNA complexes involved in nuclear import. *Journal of Molecular Biology* **271**, 718–727.

Citovsky V, Kozlovsky SV, Lacroix B, Zaltsman A, Dafny-Yelin M, Vyas S, Tovkach A, Tzfira T (2007) Biological systems of the host cell involved in *Agrobacterium* infection. *Cellular Microbiology* **9**, 9–20.

Clauce-Coupel H, Chateau S, Ducrocq C, Niot V, Kaveri S, Dubois F, Sangwan-Norreel B, Sangwan RS (2008) Role of vitronectin-like protein in *Agrobacterium* attachment and transformation of *Arabidopsis* cells. *Protoplasma* **234**, 65–75.

Colcombet J, Hirt H (2008) *Arabidopsis* MAPKs: a complex signalling network involved in multiple biological processes. *Biochemical Journal* **413**, 217–226.

Connelly C, Hieter P (1996) Budding yeast SKP1 encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. *Cell* **86**, 275–285.

Dafny-Yelin M, Levy A, Tzfira T (2008) The ongoing saga of *Agrobacterium*-host interactions. *Trends in Plant Science* **13**, 102–105.

Danhorn T, Fuqua C (2007) Biofilm formation by plant-associated bacteria. *Annual Review of Microbiology* **61**, 401–422.

De Buck S, Jacobs A, Van Montagu M, Depicker A (1999) The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. *Plant Journal* **20**, 295–304.

De Cleene M, De Ley J (1976) The host range of crown gall. *Botany Review* **42**, 389–466.

de Groot MJ, Bundock P, Hooykaas PJ, Beijersbergen AG (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnology* **16**, 839–842.

de Iannino NI, Ugalde RA (1989) Biochemical characterization of avirulent *Agrobacterium tumefaciens* chvA mutants: synthesis and excretion of beta-(1-2)glucan. *Journal of Bacteriology* **171**, 2842–2849.

De Neve M, De Buck S, Jacobs A, Van Montagu M, 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 Journal* **11**, 15–29.

Deng W, Chen L, Wood DW, Metcalfe T, Liang X, Gordon MP, Comai L, Nester EW (1998) *Agrobacterium* VirD2 protein interacts with plant host cyclophilins. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 7040–7045.

Deshaies RJ (1999) SCF and Cullin/Ring H2-based ubiquitin ligases. *Annual Review of Cell and Developmental Biology* **15**, 435–467.

Ding SW, Voinnet O (2007) Antiviral immunity directed by small RNAs. *Cell* **130**, 413–426.

Ding Z, Atmakuri K, Christie PJ (2003) The outs and ins of bacterial type IV secretion substrates. *Trends in Microbiology* **11**, 527–535.

Ditt RF, Nester EW, Comai L (2001) Plant gene expression response to *Agrobacterium tumefaciens*. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 10954–10959.

Ditt RF, Nester E, Comai L (2005) The plant cell defense and *Agrobacterium tumefaciens*. *FEMS Microbiology Letters* **247**, 207–213.

Ditt RF, Kerr KF, de Figueiredo P, Delrow J, Comai L, Nester EW (2006) The *Arabidopsis thaliana* transcriptome in response to *Agrobacterium tumefaciens*. *Molecular Plant-Microbe Interactions* **19**, 665–681.

Djamei A, Pitzschke A, Nakagami H, Rajh I, Hirt H (2007) Trojan horse strategy in *Agrobacterium* transformation: abusing MAPK defense signaling. *Science* **318**, 453–456.

Dominguez A, Fagoaga C, Navarro L, Moreno P, Pena L (2002) Regeneration of transgenic citrus plants under non selective conditions results in high-frequency recovery of plants with silenced transgenes. *Molecular Genetics and Genomics* **267**, 544–556.

Dumas F, Duckely M, Pelczar P, Van Gelder P, Hohn B (2001) An *Agrobacterium* VirE2 channel for transferred-DNA transport into plant cells. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 485–490.

Dunoyer P, Himber C, Voinnet O (2006) Induction, suppression and requirement of RNA silencing pathways in virulent *Agrobacterium tumefaciens* infections. *Nature Genetics* **38**, 258–263.

Durrenberger F, Crameri A, Hohn B, Koukolikova-Nicola Z (1989) Covalently bound VirD2 protein of *Agrobacterium tumefaciens* protects the T-DNA from exonucleolytic degradation. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 9154–9158.

Dworetzky SI, Feldherr CM (1988) Translocation of RNA-coated gold particles through the nuclear pores of oocytes. *Journal of Cell Biology* **106**, 575–584.

Endo M, Ishikawa Y, Osakabe K, Nakayama S, Kaya H, Araki T, Shibahara K, Abe K, Ichikawa H, Valentine L, Hohn B, Toki S (2006) Increased frequency of homologous recombination and T-DNA integration in *Arabidopsis* CAF-1 mutants. *EMBO Journal* **25**, 5579–5590.

Escobar MA, Dandekar AM (2003) *Agrobacterium tumefaciens* as an agent of disease. *Trends in Plant Science* **8**, 380–386.

Escudero J, Neuhaus G, Hohn B (1995) Intracellular *Agrobacterium* can transfer DNA to the cell nucleus of the host plant. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 230–234.

Essers J, Hendriks RW, Swagemakers SM, Troelstra C, de Wit J, Bootsma D, Hoeijmakers JH, Kanaar R (1997) Disruption of mouse RAD54 reduces ionizing radiation resistance and homologous recombination. *Cell* **89**, 195–204.

Forbes DJ (1992) Structure and function of the nuclear pore complex. *Annual Review of Cell Biology* **8**, 495–527.

Frary A, Hamilton CM (2001) Efficiency and stability of high molecular weight DNA transformation: an analysis in tomato. *Transgenic Research* **10**, 121–132.

Friesner J, Britt AB (2003) Ku80- and DNA ligase IV-deficient plants are sensitive to ionizing radiation and defective in T-DNA integration. *Plant Journal* **34**, 427–440.

Gallego ME, Bleuyard JY, Daoudal-Cotterell S, Jallut N, White CI (2003) Ku80 plays a role in non-homologous recombination but is not required for T-DNA integration in *Arabidopsis*. *Plant Journal* **35**, 557–565.

Gao R, Lynn DG (2005) Environmental pH sensing: resolving the VirA/VirG two-component system inputs for *Agrobacterium* pathogenesis. *Journal of Bacteriology* **187**, 2182–2189.

Garcia-Rodriguez FM, Schrammeijer B, Hooykaas PJ (2006) The *Agrobacterium* VirE3 effector protein: a potential plant transcriptional activator. *Nucleic Acids Research* **34**, 6496–6504.

Gaspar YM, Nam J, Schultz CJ, Lee LY, Gilson PR, Gelvin SB, Bacic A (2004) Characterization of the *Arabidopsis* lysine-rich arabinogalactan-protein AtAGP17 mutant (rat1) that results in a decreased efficiency of *Agrobacterium* transformation. *Plant Physiology* **135**, 2162–2171.

Gelvin SB (1998) *Agrobacterium* VirE2 proteins can form a complex with T strands in the plant cytoplasm. *Journal of Bacteriology* **180**, 4300–4302.

Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiology and Molecular Biology Reviews* **67**, 16–37.

Gheysen G, Villarroel R, Van Montagu M (1991) Illegitimate recombination in plants: a model for T-DNA integration. *Genes and Development* **5**, 287–297.

Goodner B, Hinkle G, Gattung S, Miller N, Blanchard M, Qurollo B, Goldman BS, Cao Y, Askenazi M, Halling C, Mullin L, Houmiel K, Gordon J, Vaudin M, Iartchouk O, Epp A, Liu F, Wollam C, Allinger M, Doughty D, Scott C, Lappas C, Markelz B, Flanagan C, Crowell C, Gurson J, Lomo C, Sear C, Strub G, Cielo C, Slater S (2001) Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* **294**, 2323–2328.

Grange W, Duckely M, Husale S, Jacob S, Engel A, Hegner M (2008) VirE2: a unique ssDNA-compacting molecular machine. *PLoS Biology* **6**, e44.

Hamilton CM, Frary A, Lewis C, Tanksley SD (1996) Stable transfer of intact high molecular weight DNA into plant chromosomes. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 9975–9979.

Herrera-Estrella A, Van Montagu M, Wang K (1990) A bacterial peptide acting as a plant nuclear targeting signal: the amino-terminal portion of *Agrobacterium* VirD2 protein directs a beta-galactosidase fusion protein into tobacco nuclei. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 9534–9537.

Hirooka T, Kado CI (1986) Location of the right boundary of the virulence region on *Agrobacterium tumefaciens* plasmid pTiC58 and a host-specifying gene next to the boundary. *Journal of Bacteriology* **168**, 237–243.

Hirsch AM (1999) Role of lectins (and rhizobial exopolysaccharides) in legume nodulation. *Current Opinion in Plant Biology* **2**, 320–326.

Hodges LD, Cuperus J, Ream W (2004) *Agrobacterium rhizogenes* GALLS protein substitutes for *Agrobacterium tumefaciens* single-stranded DNA-binding protein VirE2. *Journal of Bacteriology* **186**, 3065–3077.

Hodges LD, Vergunst AC, Neal-McKinney J, den Dulk-Ras A, Moyer DM, Hooykaas PJ, Ream W (2006) *Agrobacterium rhizogenes* GALLS protein contains domains for ATP binding, nuclear localization, and type IV secretion. *Journal of Bacteriology* **188**, 8222–8230.

Hodges LD, Lee LY, McNett H, Gelvin SB, Ream W (2009) The *Agrobacterium rhizogenes* GALLS gene encodes two secreted proteins required for genetic transformation of plants. *Journal of Bacteriology* **191**, 355–364.

Howard EA, Zupan JR, Citovsky V, Zambryski PC (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.

Hwang HH, Gelvin SB (2004) Plant proteins that interact with VirB2, the *Agrobacterium tumefaciens* pilin protein, mediate plant transformation. *Plant Cell* **16**, 3148–3167.

Jakubowski SJ, Cascales E, Krishnamoorthy V, Christie PJ (2005) *Agrobacterium tumefaciens* VirB9, an outer-membrane-associated component of a type IV secretion system, regulates substrate selection and T-pilus biogenesis. *Journal of Bacteriology* **187**, 3486–3495.

Jasper F, Koncz C, Schell J, Steinbiss HH (1994) *Agrobacterium* T-strand production in vitro: sequence-specific cleavage and 5′ protection of single-stranded DNA templates by purified VirD2 protein. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 694–698.

Joubert P, Beaupere D, Wadouachi A, Chateau S, Sangwan RS, Sangwan-Norreel BS (2004) Effect of phenolic glycosides on *Agrobacterium tumefaciens* virH gene induction and plant transformation. *Journal of Natural Products* **67**, 348–351.

Kado CI (2000) The role of the T-pilus in horizontal gene transfer and tumorigenesis. *Current Opinion in Microbiology* **3**, 643–648.

Kim SI, Veena V, Gelvin SB (2007) Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the *Arabidopsis* genome generated under non-selective conditions. *Plant Journal* **51**, 779–791.

Kipreos ET, Pagano M (2000) The F-box protein family. *Genome Biology* **1**, REVIEWS3002.

Klee HJ, White FF, Iyer VN, Gordon MP, Nester EW (1983) Mutational analysis of the virulence region of an *Agrobacterium tumefaciens* Ti plasmid. *Journal of Bacteriology* **153**, 878–883.

Kunik T, Tzfira T, Kapulnik Y, Gafni Y, Dingwall C, Citovsky V (2001) Genetic transformation of HeLa cells by *Agrobacterium*. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 1871–1876.

Lacroix B, Vaidya M, Tzfira T, Citovsky V (2005) The VirE3 protein of *Agrobacterium* mimics a host cell function required for plant genetic transformation. *EMBO Journal* **24**, 428–437.

Lacroix B, Li J, Tzfira T, Citovsky V (2006a) Will you let me use your nucleus? How *Agrobacterium* gets its T-DNA expressed in the host plant cell. *Canadian Journal of Physiology and Pharmacology* **84**, 333–345.

Lacroix B, Tzfira T, Vainstein A, Citovsky V (2006b) A case of promiscuity: *Agrobacterium*'s endless hunt for new partners. *Trends in Genetics* **22**, 29–37.

Lacroix B, Loyter A, Citovsky V (2008) Association of the *Agrobacterium* T-DNA-protein complex with plant nucleosomes. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 15429–15434.

Lee K, Dudley MW, Hess KM, Lynn DG, Joerger RD, Binns AN (1992) Mechanism of activation of *Agrobacterium* virulence genes: identification of phenol-binding proteins. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 8666–8670.

Lee YW, Jin S, Sim WS, Nester EW (1995) Genetic evidence for direct sensing of phenolic compounds by the VirA protein of *Agrobacterium tumefaciens*. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 12245–12249.

Lee YW, Jin S, Sim WS, Nester EW (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.

Leonetti JP, Mechti N, Degols G, Gagnor C, Lebleu B (1991) Intracellular distribution of microinjected antisense oligonucleotides. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 2702–2706.

Leskov KS, Criswell T, Antonio S, Li J, Yang CR, Kinsella TJ, Boothman DA (2001) When X-ray-inducible proteins meet DNA double strand break repair. *Seminars in Radiation Oncology* **11**, 352–372.

Levy A, Dafny-Yelin M, Tzfira T (2008) Attacking the defenders: plant viruses fight back. *Trends in Microbiology* **16**, 194–197.

Li WX, Ding SW (2001) Viral suppressors of RNA silencing. *Current Opinion in Biotechnology* **12**, 150–154.

Li J, Krichevsky A, Vaidya M, Tzfira T, Citovsky V (2005a) Uncoupling of the functions of the *Arabidopsis* VIP1 protein in transient and stable plant genetic transformation by *Agrobacterium*. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 5733–5738.

Li J, Vaidya M, White C, Vainstein A, Citovsky V, Tzfira T (2005b) Involvement of KU80 in T-DNA integration in plant cells. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 19231–19236.

Liu P, Nester EW (2006) Indoleacetic acid, a product of transferred DNA, inhibits vir gene expression and growth of *Agrobacterium tumefaciens* C58. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 4658–4662.

Llosa M, Gomis-Ruth FX, Coll M, de la Cruz Fd F (2002) Bacterial conjugation: a two-step mechanism for DNA transport. *Molecular Microbiology* **45**, 1–8.

Loake G, Grant M (2007) Salicylic acid in plant defence—the players and protagonists. *Current Opinion in Plant Biology* **10**, 466–472.

Loyter A, Rosenbluh J, Zakai N, Li J, Kozlovsky SV, Tzfira T, Citovsky V (2005) The plant VirE2 interacting protein 1. A molecular link between the *Agrobacterium* T-complex and the host cell chromatin? *Plant Physiology* **138**, 1318–1321.

Lukacs GL, Haggie P, Seksek O, Lechardeur D, Freedman N, Verkman AS (2000) Size-dependent DNA mobility in cytoplasm and nucleus. *Journal of Biological Chemistry* **275**, 1625–1629.

Matthysse AG (1983) Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection. *Journal of Bacteriology* **154**, 906–915.

Matthysse AG (1987) Characterization of nonattaching mutants of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **169**, 313–323.

Matthysse AG, McMahan S (1998) Root colonization by *Agrobacterium tumefaciens* is reduced in cel, attB, attD, and attR mutants. *Applied and Environmental Microbiology* **64**, 2341–2345.

Matthysse AG, Holmes KV, Gurlitz RH (1981) Elaboration of cellulose fibrils by *Agrobacterium tumefaciens* during attachment to carrot cells. *Journal of Bacteriology* **145**, 583–595.

Matthysse AG, Yarnall HA, Young N (1996) Requirement for genes with homology to ABC transport systems for attachment and virulence of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **178**, 5302–5308.

Matthysse AG, Marry M, Krall L, Kaye M, Ramey BE, Fuqua C, White AR (2005) The effect of cellulose overproduction on binding and biofilm formation on roots by *Agrobacterium tumefaciens*. *Molecular Plant-Microbe Interactions* **18**, 1002–1010.

Mayerhofer R, Koncz-Kalman Z, Nawrath C, Bakkeren G, Crameri A, Angelis K, Redei GP, Schell J, Hohn B, Koncz C (1991) T-DNA integration: a mode of illegitimate recombination in plants. *EMBO Journal* **10**, 697–704.

McCullen CA, Binns AN (2006) *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. *Annual Review of Cell and Developmental Biology* **22**, 101–127.

McLean BG, Greene EA, Zambryski PC (1994) Mutants of *Agrobacterium* VirA that activate vir gene expression in the absence of the inducer acetosyringone. *Journal of Biological Chemistry* **269**, 2645–2651.

Melanson D, Chilton MD, Masters-Moore D, Chilton WS (1997) A deletion in an indole synthase gene is responsible for the DIMBOA-deficient phenotype of bxbx maize. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 13345–13350.

Melchers LS, Regensburg-Tuink AJ, Schilperoort RA, Hooykaas PJ (1989a) Specificity of signal molecules in the activation of *Agrobacterium* virulence gene expression. *Molecular Microbiology* **3**, 969–977.

Melchers LS, Regensburg-Tuink TJ, Bourret RB, Sedee NJ, Schilperoort RA, Hooykaas PJ (1989b) Membrane topology and functional analysis of the sensory protein VirA of *Agrobacterium tumefaciens*. *EMBO Journal* **8**, 1919–1925.

Melchers LS, Maroney MJ, den Dulk-Ras A, Thompson DV, van Vuuren HA, Schilperoort RA, Hooykaas PJ (1990) Octopine and nopaline strains of *Agrobacterium tumefaciens* differ in virulence: molecular characterization of the virF locus. *Plant Molecular Biology* **14**, 249–259.

Michielse CB, Hooykaas PJ, Van Den Hondel CA, Ram AF (2005) *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. *Current Genetics* **48**, 1–17.

Mysore KS, Kumar CT, Gelvin SB (2000a) *Arabidopsis* ecotypes and mutants that are recalcitrant to *Agrobacterium* root transformation are susceptible to germ-line transformation. *Plant Journal* **21**, 9–16.

Mysore KS, Nam J, Gelvin SB (2000b) An *Arabidopsis* histone H2A mutant is deficient in *Agrobacterium* T-DNA integration. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 948–953.

Nagai H, Roy CR (2003) Show me the substrates: modulation of host cell function by type IV secretion systems. *Cellular Microbiology* **5**, 373–383.

Nair GR, Liu Z, Binns AN (2003) Reexamining the role of the accessory plasmid pAtC58 in the virulence of *Agrobacterium tumefaciens* strain C58. *Plant Physiology* **133**, 989–999.

Nam J, Mysore KS, Zheng C, Knue MK, Matthysse AG, Gelvin SB (1999) Identification of T-DNA tagged *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium*. *Molecular and General Genetics* **261**, 429–438.

Newell CA (2000) Plant transformation technology. Developments and applications. *Molecular Biotechnology* **16**, 53–65.

Nonaka S, Sugawara M, Minamisawa K, Yuhashi KI, Ezura H (2008a) 1-Aminocyclopropane-1-carboxylate deaminase-producing *Agrobacterium tumefaciens* has higher ability for gene transfer into plant cells. *Applied and Environmental Microbiology* **74**, 2526–2528.

Nonaka S, Yuhashi KI, Takada K, Sugaware M, Minamisawa K, Ezura H (2008b) Ethylene production in plants during transformation suppresses vir gene expression in *Agrobacterium tumefaciens*. *New Phytologist* **178**, 647–656.

Otten L, De Greve H, Leemans J, Hain R, Hooykaas PJ, Schell FM (1984) Restoration of virulence of *vir* region mutants of *A. tumefaciens* strain B6S3 by coinfection with normal and mutants *Agrobacterium* strains. *Molecular and General Genetics* **195**, 159–163.

Palmer AG, Gao R, Maresh J, Erbil WK, Lynn DG (2004) Chemical biology of multi-host/pathogen interactions: chemical perception and metabolic complementation. *Annual Review of Phytopathology* **42**, 439–464.

Pansegrau W, Schoumacher F, Hohn B, Lanka E (1993) Site-specific cleavage and joining of single-stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: analogy to bacterial conjugation. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 11538–11542.

Parke D, Ornston LN, Nester EW (1987) Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti plasmid in *Agrobacterium tumefaciens*. *Journal of Bacteriology* **169**, 5336–5338.

Peng WT, Lee YW, Nester EW (1998) The phenolic recognition profiles of the *Agrobacterium tumefaciens* VirA protein are broadened by a high level of the sugar binding protein ChvE. *Journal of Bacteriology* **180**, 5632–5638.

Peralta EG, Ream LW (1985) T-DNA border sequences required for crown gall tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 5112–5116.

Piers KL, Heath JD, Liang X, Stephens KM, Nester EW (1996) *Agrobacterium tumefaciens*-mediated transformation of yeast. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 1613–1618.

Pruss GJ, Nester EW, Vance V (2008) Infiltration with *Agrobacterium tumefaciens* induces host defense and development-dependent responses in the infiltrated zone. *Molecular Plant-Microbe Interactions* **21**, 1528–1538.

Reavy B, Bagirova S, Chichkova NV, Fedoseeva SV, Kim SH, Vartapetian AB, Taliansky ME (2007) Caspase-resistant VirD2 protein provides enhanced gene delivery and expression in plants. *Plant Cell Reports* **26**, 1215–1219.

Regensburg-Tuink AJ, Hooykaas PJ (1993) Transgenic *N. glauca* plants expressing bacterial virulence gene *vir*F are converted into hosts for nopaline strains of *A. tumefaciens*. *Nature* **363**, 69–71.

Relic B, Andjelkovic M, Rossi L, Nagamine Y, Hohn B (1998) Interaction of the DNA modifying proteins VirD1 and VirD2 of *Agrobacterium tumefaciens*: analysis by subcellular localization in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 9105–9110.

Robertson JL, Holliday T, Matthysse AG (1988) Mapping of *Agrobacterium tumefaciens* chromosomal genes affecting cellulose synthesis and bacterial attachment to host cells. *Journal of Bacteriology* **170**, 1408–1411.

Rodriguez-Navarro DN, Dardanelli MS, Ruiz-Sainz JE (2007) Attachment of bacteria to the roots of higher plants. *FEMS Microbiology Letters* **272**, 127–136.

Sagulenko E, Sagulenko V, Chen J, Christie PJ (2001) Role of *Agrobacterium* VirB11 ATPase in T-pilus assembly and substrate selection. *Journal of Bacteriology* **183**, 5813–5825.

Sahi SV, Chilton MD, Chilton WS (1990) Corn metabolites affect growth and virulence of *Agrobacterium tumefaciens*. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 3879–3883.

Salman H, Abu-Arish A, Oliel S, Loyter A, Klafter J, Granek R, Elbaum M (2005) Nuclear localization signal peptides induce molecular delivery along microtubules. *Biophysical Journal* **89**, 2134–2145.

Salomon S, Puchta H (1998) Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. *EMBO Journal* **17**, 6086–6095.

Scheiffele P, Pansegrau W, Lanka E (1995) Initiation of *Agrobacterium tumefaciens* T-DNA processing. Purified proteins VirD1 and VirD2 catalyze site- and strand-specific cleavage of superhelical T-border DNA in vitro. *Journal of Biological Chemistry* **270**, 1269–1276.

Schneeberger RG, Zhang K, Tatarinova T, Troukhan M, Kwok SF, Drais J, Klinger K, Orejudos F, Macy K, Bhakta A, Burns J, Subramanian G, Donson J, Flavell R, Feldmann KA (2005) *Agrobacterium* T-DNA integration in *Arabidopsis* is correlated with DNA sequence compositions that occur frequently in gene promoter regions. *Functional and Integrative Genomics* **5**, 240–253.

Schrammeijer B, Risseeuw E, Pansegrau W, Regensburg-Tuink TJ, Crosby WL, Hooykaas PJ (2001) Interaction of the virulence protein VirF of *Agrobacterium tumefaciens* with plant homologs of the yeast Skp1 protein. *Current Biology* **11**, 258–262.

Schrammeijer B, den Dulk-Ras A, Vergunst AC, Jurado Jacome E, Hooykaas PJ (2003) Analysis of Vir protein translocation from *Agrobacterium tumefaciens* using *Saccharomyces cerevisiae* as a model: evidence for transport of a novel effector protein VirE3. *Nucleic Acids Research* **31**, 860–868.

Sen P, Pazour GJ, Anderson D, Das A (1989) Cooperative binding of *Agrobacterium tumefaciens* VirE2 protein to single-stranded DNA. *Journal of Bacteriology* **171**, 2573–2580.

Shaked H, Melamed-Bessudo C, Levy AA (2005) High-frequency gene targeting in *Arabidopsis* plants expressing the yeast *RAD54* gene. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 12265–12269.

Shimoda N, Toyoda-Yamamoto A, Nagamine J, Usami S, Katayama M, Sakagami Y, Machida Y (1990) Control of expression of *Agrobacterium vir* genes by synergistic actions of phenolic signal molecules and monosaccharides. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 6684–6688.

Shimoda N, Toyoda-Yamamoto A, Aoki S, Machida Y (1993) Genetic evidence for an interaction between the VirA sensor protein and the ChvE sugar-binding protein of *Agrobacterium*. *Journal of Biological Chemistry* **268**, 26552–26558.

Simoh S, Quintana N, Kim HK, Choi YH, Verpoorte R (2009) Metabolic changes in *Agrobacterium tumefaciens*-infected *Brassica rapa*. *Journal of Plant Physiology* **166**, 1005–1014.

Simone M, McCullen CA, Stahl LE, Binns AN (2001) The carboxy-terminus of VirE2 from *Agrobacterium tumefaciens* is required for its transport to host cells by the virB-encoded type IV transport system. *Molecular Microbiology* **41**, 1283–1293.

Slater SC, Goldman BS, Goodner B, Setubal JC, Farrand SK, Nester EW, Burr TJ, Banta L, Dickerman AW, Paulsen I, Otten L, Suen G, Welch R, Almeida NF, Arnold F, Burton OT, Du Z, Ewing A, Godsy E, Heisel S, Houmiel KL, Jhaveri J, Lu J, Miller NM, Norton S, Chen Q, Phoolcharoen W, Ohlin V, Ondrusek D, Pride N, Stricklin SL, Sun J, Wheeler C, Wilson L, Zhu H, Wood DW (2009) Genome sequences of three *Agrobacterium* biovars help elucidate the evolution of multichromosome genomes in bacteria. *Journal of Bacteriology* **191**, 2501–2511.

Smit G, Logman TJ, Boerrigter ME, Kijne JW, Lugtenberg BJ (1989) Purification and partial characterization of the *Rhizobium leguminosarum* biovar viciae Ca^{2+}-dependent adhesin, which mediates the first step in attachment of cells of the family Rhizobiaceae to plant root hair tips. *Journal of Bacteriology* **171**, 4054–4062.

Smit G, Swart S, Lugtenberg BJ, Kijne JW (1992) Molecular mechanisms of attachment of *Rhizobium* bacteria to plant roots. *Molecular Microbiology* **6**, 2897–2903.

Smith EF, Townsend CO (1907) A plant-tumor of bacterial origin. *Science* **25**, 671–673.

Sonti RV, Chiurazzi M, Wong D, Davies CS, Harlow GR, Mount DW, Signer ER (1995) *Arabidopsis* mutants deficient in T-DNA integration. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 11786–11790.

Stachel SE, Nester EW (1986) The genetic and transcriptional organization of the vir region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. *EMBO Journal* **5**, 1445–1454.

Stachel SE, An G, Flores C, Nester EW (1985a) A Tn3 *lac*Z transposon for the random generation of beta-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. *EMBO Journal* **4**, 891–898.

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

Stachel SE, Nester EW, Zambryski PC (1986) A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 379–383.

Sung P, Krejci L, Van Komen S, Sehorn MG (2003) Rad51 recombinase and recombination mediators. *Journal of Biological Chemistry* **278**, 42729–42732.

Swart S, Logman TJ, Smit G, Lugtenberg BJ, Kijne JW (1994) Purification and partial characterization of a glycoprotein from pea (*Pisum sativum*) with receptor activity for rhicadhesin, an attachment protein of Rhizobiaceae. *Plant Molecular Biology* **24**, 171–183.

Tan TL, Kanaar R, Wyman C (2003) Rad54, a Jack of all trades in homologous recombination. *DNA Repair (Amst)* **2**, 787–794.

Tao Y, Rao PK, Bhattacharjee S, Gelvin SB (2004) Expression of plant protein phosphatase 2C interferes with nuclear import of the *Agrobacterium* T-complex protein VirD2. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 5164–5169.

Terada R, Urawa H, Inagaki Y, Tsugane K, Iida S (2002) Efficient gene targeting by homologous recombination in rice. *Nature Biotechnology* **20**, 1030–1034.

Tinland B (1996) The integration of T-DNA into plant genomes. *Trends in Plant Science* **1**, 178–184.

Tinland B, Koukolikova-Nicola Z, Hall MN, Hohn B (1992) The T-DNA-linked VirD2 protein contains two distinct functional nuclear localization signals. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 7442–7446.

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

Turk SC, van Lange RP, Regensburg-Tuink TJ, Hooykaas PJ (1994) Localization of the VirA domain involved in acetosyringone-mediated vir gene induction in *Agrobacterium tumefaciens*. *Plant Molecular Biology* **25**, 899–907.

Tzfira T (2006) On tracks and locomotives: the long route of DNA to the nucleus. *Trends in Microbiology* **14**, 61–63.

Tzfira T, Citovsky V (2001) Comparison between nuclear import of nopaline- and octopine-specific VirE2 protein of *Agrobacterium* in plant and animal cells. *Molecular Plant Pathology* **2**, 171–176.

Tzfira T, Vaidya M, Citovsky V (2001) VIP1, an *Arabidopsis* protein that interacts with *Agrobacterium* VirE2, is involved in VirE2 nuclear import and *Agrobacterium* infectivity. *EMBO Journal* **20**, 3596–3607.

Tzfira T, Vaidya M, Citovsky V (2002) Increasing plant susceptibility to *Agrobacterium* infection by overexpression of the *Arabidopsis* nuclear protein VIP1. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 10435–10440.

Tzfira T, Frankman LR, Vaidya M, Citovsky V (2003) Site-specific integration of *Agrobacterium tumefaciens* T-DNA via double-stranded intermediates. *Plant Physiology* **133**, 1011–1023.

Tzfira T, Li J, Lacroix B, Citovsky V (2004a) *Agrobacterium* T-DNA integration: molecules and models. *Trends in Genetics* **20**, 375–383.

Tzfira T, Vaidya M, Citovsky V (2004b) Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*. *Nature* **431**, 87–92.

Usui T, Ohta T, Oshiumi H, Tomizawa J, Ogawa H, Ogawa T (1998) Complex formation and functional versatility of Mre11 of budding yeast in recombination. *Cell* **95**, 705–716.

van Attikum H, Hooykaas PJ (2003) Genetic requirements for the targeted integration of *Agrobacterium* T-DNA in *Saccharomyces cerevisiae*. *Nucleic Acids Research* **31**, 826–832.

van Attikum H, Bundock P, Hooykaas PJ (2001) Non-homologous end-joining proteins are required for *Agrobacterium* T-DNA integration. *EMBO Journal* **20**, 6550–6558.

Veena, Jiang H, Doerge RW, Gelvin SB (2003) Transfer of T-DNA and Vir proteins to plant cells by *Agrobacterium tumefaciens* induces expression of host genes involved in mediating transformation and suppresses host defense gene expression. *Plant Journal* **35**, 219–236.

Vergunst AC, Schrammeijer B, den Dulk-Ras A, de Vlaam CM, Regensburg-Tuink TJ, Hooykaas PJ (2000) VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. *Science* **290**, 979–982.

Vergunst AC, van Lier MC, den Dulk-Ras A, Hooykaas PJ (2003) Recognition of the *Agrobacterium tumefaciens* VirE2 translocation signal by the VirB/D4 transport system does not require VirE1. *Plant Physiology* **133**, 978–988.

Vergunst AC, van Lier MC, den Dulk-Ras A, Stuve TA, Ouwehand A, Hooykaas PJ (2005) Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of *Agrobacterium*. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 832–837.

Verkman AS (2002) Solute and macromolecule diffusion in cellular aqueous compartments. *Trends in Biochemical Sciences* **27**, 27–33.

Voinnet O, Rivas S, Mestre P, Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant Journal* **33**, 949–956.

Wagner VT, Matthysse AG (1992) Involvement of a vitronectin-like protein in attachment of *Agrobacterium tumefaciens* to carrot suspension culture cells. *Journal of Bacteriology* **174**, 5999–6003.

Walker TS, Bais HP, Grotewold E, Vivanco JM (2003) Root exudation and rhizosphere biology. *Plant Physiology* **132**, 44–51.

Wang K, Stachel SE, Timmerman B, Van Montagu M, Zambryski PC (1987) Site-specific nick in the T-DNA border sequence as a result of *Agrobacterium* vir gene expression. *Science* **235**, 587–591.

Wang MB, Metzlaff M (2005) RNA silencing and antiviral defense in plants. *Current Opinion in Plant Biology* **8**, 216–222.

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

Winans SC (1990) Transcriptional induction of an *Agrobacterium* regulatory gene at tandem promoters by plant-released phenolic compounds, phosphate starvation, and acidic growth media. *Journal of Bacteriology* **172**, 2433–2438.

Winans SC, Kerstetter RA, Nester EW (1988) Transcriptional regulation of the virA and virG genes of *Agrobacterium tumefaciens*. *Journal of Bacteriology* **170**, 4047–4054.

Windels P, De Buck S, Van Bockstaele E, De Loose M, Depicker A (2003) T-DNA integration in *Arabidopsis* chromosomes. Presence and origin of filler DNA sequences. *Plant Physiology* **133**, 2061–2068.

Wood DW, Setubal JC, Kaul R, Monks DE, Kitajima JP, Okura VK, Zhou Y, Chen L, Wood GE, Almeida NF Jr., Woo L, Chen Y, Paulsen IT, Eisen JA, Karp PD, Bovee D Sr., Chapman P, Clendenning J, Deatherage G, Gillet W, Grant C, Kutyavin T, Levy R, Li MJ, McClelland E, Palmieri A, Raymond C, Rouse G, Saenphimmachak C, Wu Z, Romero P, Gordon D, Zhang S, Yoo H, Tao Y, Biddle P, Jung M, Krespan W, Perry M, Gordon-Kamm B, Liao L, Kim S, Hendrick C, Zhao ZY, Dolan M, Chumley F, Tingey SV, Tomb JF, Gordon MP, Olson MV, Nester EW (2001) The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* **294**, 2317–2323.

Wright EL, Deakin WJ, Shaw CH (1998) A chemotaxis cluster from *Agrobacterium tumefaciens*. *Gene* **220**, 83–89.

Yi H, Mysore KS, Gelvin SB (2002) Expression of the *Arabidopsis* histone *H2A-1* gene correlates with susceptibility to *Agrobacterium* transformation. *Plant Journal* **32**, 285–298.

Young C, Nester EW (1988) Association of the virD2 protein with the 5′ end of T strands in *Agrobacterium tumefaciens*. *Journal of Bacteriology* **170**, 3367–3374.

Yuan ZC, Edlind MP, Liu P, Saenkham P, Banta LM, Wise AA, Ronzone E, Binns AN, Kerr K, Nester EW (2007) The plant signal salicylic acid shuts down expression of the *vir* regulon and activates quormone-quenching genes in *Agrobacterium*. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 11790–11795.

Zhang J, Boone L, Kocz R, Zhang C, Binns AN, Lynn DG (2000) At the maize/*Agrobacterium* interface: natural factors limiting host transformation. *Chemistry and Biology* **7**, 611–621.

Zhou XR, Christie PJ (1997) Suppression of mutant phenotypes of the *Agrobacterium tumefaciens* VirB11 ATPase by overproduction of VirB proteins. *Journal of Bacteriology* **179**, 5835–5842.

Zhu Y, Nam J, Carpita NC, Matthysse AG, Gelvin SB (2003) *Agrobacterium*-mediated root transformation is inhibited by mutation of an *Arabidopsis* cellulose synthase-like gene. *Plant Physiology* **133**, 1000–1010.

Ziemienowicz A, Gorlich D, Lanka E, Hohn B, Rossi L (1999) Import of DNA into mammalian nuclei by proteins originating from a plant pathogenic bacterium. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 3729–3733.

Ziemienowicz A, Tinland B, Bryant J, Gloeckler V, Hohn B (2000) Plant enzymes but not *Agrobacterium* VirD2 mediate T-DNA ligation in vitro. *Molecular and Cellular Biology* **20**, 6317–6322.

Ziemienowicz A, Merkle T, Schoumacher F, Hohn B, Rossi L (2001) Import of *Agrobacterium* T-DNA into plant nuclei: two distinct functions of VirD2 and VirE2 proteins. *Plant Cell* **13**, 369–383.

Zupan JR, Citovsky V, Zambryski P (1996) *Agrobacterium* VirE2 protein mediates nuclear uptake of single-stranded DNA in plant cells. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 2392–2397.

Zupan JR, Ward D, Zambryski P (1998) Assembly of the VirB transport complex for DNA transfer from *Agrobacterium tumefaciens* to plant cells. *Current Opinion in Microbiology* **1**, 649–655.