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The roles of bacterial and host plant factors in *Agrobacterium*-mediated genetic transformation

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Abstract

The genetic transformation of plants mediated by *Agrobacterium tumefaciens* represents an essential tool for both fundamental and applied research in plant biology. For a successful infection, culminating in the integration of its transferred DNA (T-DNA) into the host genome, *Agrobacterium* relies on multiple interactions with host-plant factors. Extensive studies have unraveled many of such interactions at all major steps of the infection process: activation of the bacterial virulence genes, cell-cell contact and macromolecular translocation from *Agrobacterium* to host cell cytoplasm, intracellular transit of T-DNA and associated proteins (T-complex) to the host cell nucleus, disassembly of the T-complex, T-DNA integration, and expression of the transferred genes. During all these processes, *Agrobacterium* has evolved to control and even utilize several pathways of host-plant defense response. Studies of these *Agrobacterium*-host interactions substantially enhance our understanding of many fundamental cellular biological processes and allow improvements in the use of *Agrobacterium* as a gene transfer tool for biotechnology.

Keywords

Agrobacterium; genetic transformation; macromolecular transport; T-DNA expression

Introduction

Agrobacterium tumefaciens has served as an essential tool for research in plant biology and biotechnology in the last several decades (Newell, 2000). The exceptional ability of *Agrobacterium* to transfer a part of its own DNA to the host plant genome represents a rare case of naturally occurring horizontal gene transfer, and is the basis of its use for transgenesis (Gelvin, 2003; Tzfira and Citovsky, 2006). This capability relies on a specialized plasmid, the tumor-inducing (Ti) plasmid, that contains two essential regions required for DNA transfer to the host cell (Fig. 1). The presence of the Ti plasmid is responsible for the virulence of *Agrobacterium*, and a non-virulent strain may become virulent by acquiring this plasmid (Lacroix, 2013a). The first essential region is the

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transferred DNA (T-DNA) itself; it is delimited by two direct repeat sequences of about 25 base pairs, termed the left and right borders (LB and RB). These borders are necessary and sufficient to define a functional T-DNA element, while the transferred sequence between them may be modified at will. The T-DNA is not transported to the host plant cell as a double-stranded molecule; instead, VirD2 and VirD1, protein products of the Ti plasmid virulence region (see below), form a nuclease that nicks LB and RB, and a mobile single-stranded (ss) T-DNA form, termed the T-strand, is generated by strand replacement synthesis (Gelvin, 2003; Tzfira and Citovsky, 2006) (Fig. 1).

The second essential region, the virulence (*vir*) genes, composed of seven major loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virF*, and *virG*), encodes most of the bacterial protein machinery required for virulence (Fig. 1) (Zupan and Zambryski, 1995). In the wild-type *Agrobacterium*, the T-DNA contains about fifteen genes that are expressed in the transformed plant cells and lead to the crown-gall disease (Escobar and Dandekar, 2003; Lacroix, 2013a). A subset of the T-DNA genes encodes proteins involved in plant growth regulator synthesis and sensitivity, which induce uncontrolled host cell division and result in the visible symptoms of *Agrobacterium* infection, i.e., tumors or crown galls. Other T-DNA gene products are involved in the production of opines, small secreted molecules that *Agrobacterium* cells use as source of carbon and nitrogen (Hooykaas, 1994).

The natural host range of *Agrobacterium* in the plant Kingdom is very wide; species susceptible to *Agrobacterium* infection are found in most dicotyledonous and gymnosperm families, and in a few monocotyledonous families (De Cleene and De Ley, 1976). Under laboratory conditions, many other plant species that are not natural hosts can be made susceptible to *Agrobacterium*, for example by manipulating plant tissue culture conditions or artificially activating *Agrobacterium* virulence (Newell, 2000). However, generating stably transformed transgenic plants is still a challenge for many plant species, especially some agronomically important grains (Gelvin, 2010). In addition to plants, *Agrobacterium* mediated transformation of other eukaryotic species has been achieved under laboratory conditions (Lacroix et al., 2006): notably yeast (Bundock et al., 1995; Piers et al., 1996), many species of fungi (De Groot et al., 1998; Michielse et al., 2005), and even cultured human cells (Kunik et al., 2001).

In this article, we review the transfer of DNA between *Agrobacterium* and host cells (Fig. 1), focusing on the roles of plant factors in this process. In many ways, a better understanding of the diverse and critical functions of host factors in the transformation events may help to improve the efficiency of *Agrobacterium*-mediated genetic modification of species of interest. Moreover, *Agrobacterium* represents a unique experimental system to study the fundamental biological mechanisms and cellular systems, e.g., nuclear import machinery, chromatin targeting, proteasomal degradation, DNA repair, and regulation of transgene expression involved in genetic transformation of eukaryotic cells.

Chemical communication between *Agrobacterium* and plant cells

Because the activation of the bacterial virulence system represents a substantial investment of energy, a tight regulation of virulence induction in response to environmental stimuli

is necessary. Indeed, the rhizosphere is a complex and dynamic environment, where plant-associated microorganisms constantly perceive different biotic signals and modify their behavior accordingly (Brencic and Winans, 2005). Specifically, *Agrobacterium* detects chemical signals emitted by host plants via its cell surface sensors and responds to them by optimizing the activity of its virulence system (Fig. 2).

The main and first discovered plant factor regulating *Agrobacterium* virulence is the phenolic compound acetosyringone (3,5-dimethoxyacetophenone, AS), present in plant cell exudates and capable of inducing the *vir* gene expression even in the absence of plant cells (Bolton et al., 1986; Stachel et al., 1985). AS is recognized by the bacteria via a two-component receptor system, composed of the VirA and VirG proteins (Klee et al., 1983; Stachel and Zambryski, 1986). The sensing of AS by the VirA/VirG receptor results in strong and rapid expression of all the *vir* genes. The *virA* and *virG* genes themselves are expressed at a low basal level in absence of AS, and are also highly inducible via a self-regulated system (Winans et al., 1988). Later, it was discovered that many other phenolic compounds resembling AS, including glycoside derivatives (Joubert et al., 2004), can also activate *vir* gene expression (Melchers et al., 1989a). Common structural features between these compounds, allowing them to interact with bacterial receptor, suggest that they are recognized by a unique protein at the surface of bacterial cell (Lee et al., 1992). Genetic studies have demonstrated that AS or related compounds bind directly to the VirA protein (Lee et al., 1995); indeed, specific ranges of phenolic compounds recognized by different strains of *Agrobacterium* can be modified by exchanging their *virA* genes. Upon phenolic activator binding, VirA undergoes autophosphorylation and, in turn, phosphorylates VirG; the phosphorylated VirG targets a 12-bp long specific sequence, termed the *vir* box, present in all *vir* operon promoters, which results in transcriptional activation of all *vir* genes (Brencic and Winans, 2005). The biosynthetic pathway for AS in plants is not completely characterized, but it has been shown that plants deficient in enzymes of the phenylpropanoid pathway are less susceptible to *Agrobacterium*, most likely because of reduced production of AS or related compounds (Maury et al., 2010). Interestingly, the expression levels of several genes encoding enzymes of this pathway increase upon *Agrobacterium* infection (Ditt et al., 2006). Because phenylpropanoids are involved, among other functions, in plant defense and survival (Fraser and Chapple, 2011); *Agrobacterium* may have evolved to subvert this host defense response to enhance its infection.

In addition to phenolic compounds, other signals emitted by plants affect *vir* gene expression. Reducing sugar monomers cannot activate the virulence system alone, but they are able to enhance AS action in two ways: by enhancing the sensitivity of the VirA/G system to phenolics and by elevating the saturating concentration of phenolics for virulence activation (Cangelosi et al., 1990; Shimoda et al., 1990). Additionally, the presence of monosaccharides as coinducers may result in increasing the range of phenolics recognized by the bacterial *vir* gene induction system (Peng et al., 1998). That the known coinducer monosaccharides, such as D-glucose and D-galactose (Ankenbauer and Nester, 1990; Shimoda et al., 1990), have common structural features, i.e., a pyranose ring and acidic groups, also suggests that they may be detected by a unique specific receptor. Indeed, a bacterial chromosome-encoded periplasmic protein ChvE mediates sensing of and virulence response to monosaccharides. ChvE is thought first to bind monosaccharides, and then to

enhance the VirA activity as a *vir* gene inducer by interacting with the VirA periplasmic domain (Cangelosi et al., 1990; Lee et al., 1992; Shimoda et al., 1993).

Two conditions that are frequently observed in the rhizosphere, low pH and low phosphate concentration, are known to enhance activation of the virulence system. The effect of low pH, e.g., pH 5.7, is mediated by VirA (Chang et al., 1996; Melchers et al., 1989b) and ChvE (Gao and Lynn, 2005). Moreover, the *virG* expression is induced by low pH and low concentration of phosphate, most likely via the activation of another two-component regulatory system composed of the ChvG and ChvI proteins (Charles and Nester, 1993; Yuan et al., 2008).

Chemicals emitted by some plant species can also act as inhibitors of the *Agrobacterium* virulence, which may help explain the important interspecific variability in resistance to *Agrobacterium*. Two chemical compounds found in corn seedling homogenates were shown to have such inhibitory effect: DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) is an inhibitor of both *Agrobacterium* growth and AS-dependent virulence activation (Sahi et al., 1990), and MDIBOA (2-hydroxy-4,7-dimethoxybenzoxazin-3-one) is a potent inhibitor of *Agrobacterium* virulence with a limited effect on bacterial growth (Zhang et al., 2000). DIMBOA and MDIBOA derive from the tryptophan biosynthetic pathway (Melanson et al., 1997), like the auxin indole acetic acid (IAA). In fact, IAA itself is able to inhibit *vir* gene induction, likely by competing with the inducing phenolic compounds, such as AS, for binding to VirA (Liu and Nester, 2006). Because IAA is produced at high concentrations during development of crown gall tumors resulting from *Agrobacterium* infection, it may inhibit secondary transformation by the initial infecting bacterial strain or by another competing strain.

Two signal molecules involved in plant response to several types of biotic or abiotic stresses have also been shown to act as negative regulators of *Agrobacterium* virulence. First, the phenolic compound salicylic acid (SA), the major signal molecule of systemic acquired resistance (SAR) (Vlot et al., 2009), inhibits expression of all *vir* genes, most likely by attenuating the VirA protein kinase activity (Yuan et al., 2007). Indeed, *Arabidopsis* or tobacco mutants deficient in SA accumulation are more sensitive to *Agrobacterium* infection, and mutants overproducing SA or plant treated with exogenous SA are relatively resistant (Anand et al., 2008; Yuan et al., 2007). Second, studies of plants affected in ethylene production suggested that this plant gaseous growth regulator can inhibit *Agrobacterium* virulence (Nonaka et al., 2008b). Consistently, inducing degradation of the direct precursor of ethylene 1-aminocyclopropane-1-carboxylate (ACC) in *Agrobacterium* cells by expression of ACC deaminase enhances transformation efficiency (Nonaka et al., 2008a). Yet, a direct effect of ethylene on *vir* gene expression could not be demonstrated.

Cell-to-cell contact and attachment

Intuitively, it is logical to assume that a close contact between *Agrobacterium* cells and their host cells is required for T-DNA transfer. Indeed, *Agrobacterium* mutants affected in their ability to attach to plant cells usually show reduced virulence (Matthysse, 1987). However, whereas several candidates have been proposed for the roles of the putative plant or bacterial

cell surface receptors, to date this function has not been confirmed for any of them. Like in many other cases of plant-associated bacteria, cell-to-cell contact is believed to occur in two sequential stages (Rodriguez-Navarro et al., 2007; Tomlinson and Fuqua, 2009). First, a reversible attachment is mediated by bacterial and plant extracellular factors, adhesins; however, none of such molecules have yet been identified for the *Agrobacterium*-plant cell interaction. Second, the initial attachment is consolidated via the synthesis of bacterial exocellular glucans leading to biofilm formation (Matthysse, 1983; Tomlinson and Fuqua, 2009).

On the bacterial side, numerous genes have been proposed to play a role in the initial attachment; however, for most of them, a function could not be defined, and many of them were found to be not essential for virulence. Extracellular components of the type IV secretion system (T4SS), i.e. VirB1*, VirB2, and VirB5, are good candidates for interacting with a potential plant receptor. Specifically, VirB2 and VirB5 have been suggested to function as adhesins, based on the fact that their orthologs play this role in other pathogenic bacteria, such as *Brucella* sp. (Backert et al., 2008). However, it is still unknown whether these VirB proteins are involved in the early steps of cell-cell recognition, and their only proven classical function remains the transport of the bacterial T-DNA and effector proteins into the host cell cytoplasm. Furthermore, the available data suggest that the entire *vir* region is not essential for bacterial attachment. Another subset of the *Agrobacterium* genes, the *att* region located in the pAt linear chromosome, have initially been suspected to mediate attachment (Matthysse and McMahan, 1998); however later studies demonstrated that the *att* genes are not required for *Agrobacterium* virulence, but are mainly involved in quorum sensing (Nair et al., 2003). Finally, only three bacterial factors, i.e., the chromosome-located *chvA*, *chvB*, and *exoC* genes involved in synthesis of exocellular oligosaccharides, such as the cyclic 1,2- β -D-glucan, have been unequivocally shown to play an essential role in both attachment and virulence (Cangelosi et al., 1989; De Iannino and Ugalde, 1989). But what are the host factors that might recognize and bind these exopolysaccharides of *Agrobacterium*?

Lectins, a family of plant proteins that bind reversibly mono- or oligosaccharides, are known to interact with exopolysaccharides of several species of *Rhizobiaceae* (Hirsch, 1999). Similarly, host lectins could be involved in recognition of the *Agrobacterium* exocellular cyclic glucan produced via the *chvA*, *chvB*, and *exoC* system; as yet, however, no such lectins have been identified.

Another potential candidate for initial attachment of *Agrobacterium* to the host cell is rhicadhesin. Rhicadhesin was first identified as an extracellular protein in *Rhizobium*, and it inhibits the attachment of both *Agrobacterium* and *Rhizobium* cells to the plant cell surface, potentially by saturating a host receptor (Smit et al., 1989). However, no genes encoding rhicadhesin-like proteins are found in the *Agrobacterium* genome. In plants, putative receptors for rhicadhesin-like molecules have been identified, such as a carrot cell surface vitronectin-like protein (Wagner and Matthysse, 1992) or a pea cell wall germin-like glycoprotein (Swart et al., 1994). However, their role in bacterial attachment and actual binding to rhicadhesin have never been substantiated, and a more recent study has

demonstrated that a vitronectin-like protein present in the *Arabidopsis* cell wall is involved neither in attachment nor in virulence of *Agrobacterium* (Clauce-Coupelet et al., 2008).

A forward genetic screen for *Arabidopsis* resistant to *Agrobacterium* (*rat*) mutants led to identification of several plant lines mutated in genes encoding extracellular proteins, potentially involved in bacterial attachment. For example, the *rat4* mutant is deficient in a homolog of cellulose synthase, CSLA9, raising a possibility that modifications of the plant cell surface by CSLA9 could affect bacterial attachment (Zhu et al., 2003a). That *Agrobacterium* is also able to infect fungi and animal cells suggests that a plant-specific receptor is not absolutely required for virulence. Nevertheless, in nature, *Agrobacterium* strains able to bind to a plant receptor to enhance the cell-to-cell contact would certainly gain advantage in competing with bacterial strains that cannot recognize such receptor(s).

In the second stage, the attachment of *Agrobacterium* to host cell is consolidated, mostly by the synthesis of bacterial cellulose fibrils and cyclic glucans, which results in the formation of a biofilm in which bacteria are embedded at the plant cell surface. Biofilm formation appears essential for the virulence of most plant-associated bacteria (Danhorn and Fuqua, 2007), including *Agrobacterium* (Matthysse et al., 2005; Tomlinson et al., 2010). However, whereas *Agrobacterium* mutants disrupted in the *celABCDE* operon are unable to synthesize cellulose and are impaired in their attachment to plant cells, their tumorigenicity is only slightly diminished (Matthysse, 1983). Thus, unlike cyclic glucans (Cangelosi et al., 1989; De Iannino and Ugalde, 1989), cellulose fibrils are not absolutely required for *Agrobacterium* virulence. As mentioned above, there are no known plant factors that interact with bacterial exocellular glucans during the initial attachment stage; however, these glucans might play a role in determination of structural and chemical properties of the host cell surface that, in turn, may affect the formation of biofilms during the attachment consolidation stage.

Transferred-DNA and protein entry into host plant cell

Agrobacterium T-DNA and protein translocation into the host cell is mediated by the bacterial T4SS, composed of the eleven proteins encoded by the *virB* operon and the *virD4* gene. *Agrobacterium* T4SS is particularly well studied, and T4SS structure and the functions of its protein components are well understood (Christie, 2004), including the sequence of contacts of the T-DNA transport substrate with different subunits of T4SS (Cascales and Christie, 2004). However, the mechanism by which T4SS traverses the plant cell wall and plasma membrane for delivery of the transport substrates, i.e., the T-DNA and effector proteins, into the host cell cytoplasm remains unknown.

Because the T-pilus represents the extracellular appendage of the T4SS, the role of its components, mainly of VirB2 and VirB5, and their potential interactions with plant cell surface factors could provide clues for understanding how T4SS negotiates these cellular barriers. In a yeast-two-hybrid screen, four plant proteins interacting with the processed carboxyl terminal VirB2, i.e., VirB2 lacking the amino terminal 42 amino acid-long signal peptide cleaved before the T-pilus biogenesis, were identified (Hwang and Gelvin, 2004). Three of them are related proteins of unknown function, designated BTI1, 2, and 3, and the

fourth is a membrane-associated GTPase, AtRAB8. Plant susceptibility to *Agrobacterium* correlates with the expression levels of these proteins (Hwang and Gelvin, 2004). Thus, these proteins are good candidates for the role of plant receptors mediating interaction with the T-pilus, albeit it has not been determined whether they are involved in the early stages of the *Agrobacterium*-plant cell interaction or in the process of macromolecular translocation itself. Similarly, although the exact function of VirB5 is unknown, its extracellular localization, most likely at the tip of the T-pilus, indicates possible interactions with a host cell surface factor (Aly and Baron, 2007). Consistent with this idea, exogenous extracellular VirB5 protein enhances infectivity of the wild-type *Agrobacterium*, although it does not rescue infectivity of an *Agrobacterium* mutant disrupted in the *virB5* gene (Lacroix and Citovsky, 2011). These observations suggest a dual role for VirB5: intrabacterial function for biosynthesis and/or stability of the T-pilus, and an extracellular function. Interestingly, CagL, an ortholog of VirB5 in the animal pathogen *Helicobacter pylori*, interacts with the host integrin; whether this interaction plays a role in macromolecular translocation, or it is simply involved in triggering intracellular signaling in the host cell, remains unknown (Tegtmeier et al., 2011).

Similarly to protein translocation by type III secretion systems (Thanassi et al., 2012), macromolecules translocated by T4SS could be injected directly from the bacterial to the host cytoplasm through the T-pilus acting as a hollow needle (Kado, 2000). The T-pilus has a lumen diameter of 2 nm, compatible with the passage of folded protein and ssDNA molecules (Kado, 2000). Alternatively, the T-pilus may act by mechanically perforating the host cell wall and plasma membrane, thus allowing the entry of the transported molecules through a T4SS transport conduit (Llosa et al., 2002). That these two possible modes of macromolecule translocation do not postulate a plant-specific receptor is consistent with the *Agrobacterium*'s ability to transfer macromolecules to a wide variety of eukaryotic cells, including non-plant hosts (Lacroix et al., 2006). Another series of studies suggested that T-DNA transfer can occur even in absence of detectable levels of T-pilus biogenesis. Indeed, substrate transfer through the *Agrobacterium* T4SS is not abolished by the blocking VirB2 polymerization and, thus, inhibition of the T-pilus formation (Sagulenko et al., 2001).

A radically different mechanism invoked for the entry of T-DNA into the host cell relies upon the formation of protein channels in lipidic membrane, composed of the *Agrobacterium* VirE2 protein (Dumas et al., 2001). Such channels may allow the passage of macromolecules through host membrane. In addition, the very efficient and cooperative binding of VirE2 (Citovsky et al., 1989) to the T-strand in the host cell cytoplasm may actively pull the T-DNA molecule out of the T4SS and/or VirE2 channels, without requiring external energy sources (Grange et al., 2008). The biological relevance of these VirE2 activities for the actual transformation process remains to be demonstrated *in vivo*, and potential VirE2-interacting plant factors that may be involved in this process await to be discovered.

Nuclear import

Because the final destination of the T-DNA is the host cell nucleus, where it is to be integrated in the host genome, T-DNA targeting to the nucleus and passage through the

nuclear pore represent an important step of the genetic transformation process. The T-DNA nuclear import is mediated by bacterial effector proteins, VirD2 and VirE2, associated with the T-DNA and several host proteins that interact with these effectors. This interaction network is summarized in Fig. 3.

The bacterial endonuclease component VirD2, which is covalently associated with the 5' end of the exported T-strand molecule (Ward and Barnes, 1988; Young and Nester, 1988) (see Fig. 1), interacts directly with the plant importin α , a component of the cellular nuclear import machinery, that mediates the nuclear import of VirD2 (Ballas and Citovsky, 1997), and, by implication its associated T-strand. Two nuclear localization signals (NLSs) are found within VirD2, a monopartite amino terminal NLS and a bipartite carboxyl terminal NLS (Herrera-Estrella et al., 1990; Howard et al., 1992; Tinland et al., 1992), but only the latter is necessary for the VirD2 nuclear import (Howard et al., 1992; Ziemienowicz et al., 2001). In addition, two other plant proteins interacting with VirD2 might modulate its nuclear localization: an *Arabidopsis* cyclophilin that may further assist VirD2 nuclear import (Deng et al., 1998); and a tomato type 2C serine/threonine protein phosphatase is thought to dephosphorylate VirD2 and thereby inhibit its nuclear import, although the role of VirD2 phosphorylation in the nuclear import has not been demonstrated directly (Tao et al., 2004).

The VirE2 nuclear import is more complex. Unlike VirD2, VirE2 does not interact efficiently with importin α (Citovsky et al., 2004), although recent data have detected such interaction with one specific member of this protein family (Bhattacharjee et al., 2008) (see below). Instead, it relies on the presence of the VirE2 interacting protein 1 (VIP1), a plant basic leucine zipper (bZIP) motif protein (Tzfira et al., 2001). VIP1 has been shown to act as a molecular adapter between VirE2 and the host nuclear import machinery by binding directly to both VirE2 and importin α (Citovsky et al., 2004; Tzfira et al., 2001; 2002). Thus, both VirD2 and VirE2 are imported into the host cell nucleus via the importin α -dependent pathway, VirD2 – directly, and VirE2 – largely by piggybacking on VIP1. The nuclear localization of VIP1 itself is regulated by serine phosphorylation at position 79 by the mitogen-activated protein (MAP) kinase 3 (MPK3) (Djamei et al., 2007). MAP kinases are important signal transduction factors involved in plant responses to many biotic and abiotic stimuli (Colcombet and Hirt, 2008), and MPK3 is activated during plant defense reactions by several factors, including *Agrobacterium* infection. Moreover, an *Arabidopsis* mutant in the *MPK3* gene is resistant to *Agrobacterium* (Djamei et al., 2007). Therefore, the induction of MPK3 and subsequent phosphorylation of VIP1, which are normally part of the plant defense reaction, are subverted by *Agrobacterium* to enhance its infectivity (Djamei et al., 2007).

In general, *Agrobacterium* might “choose” different cellular pathways to achieve successful infection, which rely on bacterial or plant factors according to the host species and/or physiological conditions. For example, it has been shown that VirE2 can also interact directly with some members of the plant importin α family, such as importin α -4 (Bhattacharjee et al., 2008), which potentially assist its nuclear import in the absence, or in addition to, VIP1. Yet it remains unclear whether this interaction, indeed, is functionally important for the VirE2 nuclear import. Unlike VIP1, which can bind VirE2 when the latter

is associated with ssDNA (Lacroix et al., 2008), it remains unknown whether importin α -4 can also bind VirE2 in such nucleoprotein complex.

Interestingly, VirE3, another *Agrobacterium* virulence protein translocated into the plant cell, partially mimics the VIP1 function: it can interact with both VirE2 and importin α and facilitate the nuclear import of VirE2 (Lacroix et al., 2005). VirE3 is not absolutely essential for plant genetic transformation by *Agrobacterium*, but might compensate for the lack of VIP1-like protein in some plant species, consistent with its proposed role as a host range factor (Hirooka and Kado, 1986). VirE3 nuclear import relies on the canonical bipartite NLS (Lacroix et al., 2005); curiously, like VIP1, VirE3 has been suggested to act as transcription factor, although its potential target genes are unknown (Garcia-Rodriguez et al., 2006). Two other *Agrobacterium* virulence proteins known to be exported into the host-plant cell, VirD5 and VirF (see below), are also targeted to the nucleus (Magori and Citovsky, 2011; Tzfira et al., 2004b), likely by direct interaction with the host cell nuclear import machinery.

Soon after their entry in the host cell cytoplasm, the VirD2-T-strand complex and VirE2 molecules—thought to be translocated into the host cell independently of each other (Citovsky et al., 1992; Gelvin, 1998; Otten et al., 1984; Vergunst et al., 2000)—most likely associate to form the mature T-complex. Because the major structural components of the T-complex are the T-strand and VirE2, and because T-DNA is not sequence-specific (Zambryski, 1992), VirE2-ssDNA complexes can be considered to represent a minimal synthetic T-complex (Abu-Arish et al., 2004; Citovsky et al., 1997; Dym et al., 2008; Lacroix et al., 2008; Zupan et al., 1996). Structural analyses of these synthetic T-complexes (Abu-Arish et al., 2004; Citovsky et al., 1997; Grange et al., 2008) indicated a coiled filament with a 13-15 nm diameter (Abu-Arish et al., 2004), which is larger than the 9-nm diffusion limit of the nuclear pore (Forbes, 1992). Based on these parameters, a typical mature T-complex from a nopaline-specific *Agrobacterium* strain (Citovsky et al., 1992) would be composed of a 22-kb T-strand, 1,176 molecules of VirE2, and one molecule of VirD2, with a total molecular mass of about 90 megadaltons; the movement of such a large complex through the cytoplasm by diffusion would be very limited (Tzfira, 2005), and its passive entry into the nucleus impossible (Forbes, 1992). Therefore, nuclear import of the T-complex as well as its transcytoplasmic transport toward the nucleus most likely occur by active mechanisms. Due to the sequence non-specific nature of the T-strand, the T-complex nuclear import must rely on the nuclear targeting abilities of its protein components, VirE2 and VirD2, and thus occur via the importin α -dependent pathway. Indeed, VirD2, as well as VirE2, can mediate nuclear import of short segments of ssDNA, independently of each other, in animal (Ziemienowicz et al., 1999) and in plant cells (Gelvin, 1998; Zupan et al., 1996). Although these data suggest a redundancy between VirD2 and VirE2 roles in nuclear import of the T-complex, an efficient transport of the T-complex through the nuclear pore, which is thought to occur in a polar fashion (Tzfira et al., 2000; Zambryski, 1992), is likely to require both factors (Ziemienowicz et al., 2001). Consistent with the polar structure of the T-complex, VirD2, attached to the 5'-end of the T-strand, may direct the T-complex toward the nuclear pore, while VirE2 and its associated VIP1 or VirE3, that package the entire length of the T-strand, assist the translocation process, bringing it to completion. Lending support to this model, *Agrobacterium*-mediated genetic transformation with very long (about

150 kb) segments of DNA is enhanced by expressing additional copies of the *virE2* gene in the bacterium (Hamilton et al., 1996).

Many DNA viruses depend on molecular motors, such as dynein, and the microtubule network for their transcytoplasmic transport (Dodding and Way, 2011). Two lines of evidence suggest a similar role for molecular motors in the T-complex movement through the host cell cytoplasm. First, VIP1, a T-complex associated host protein, has been shown to interact with the dynein-like DLC3 protein of *Arabidopsis* (Tzfira, 2006). Second, the observations that synthetic T-complexes are actively transported along the microtubule network in a cell-free system suggest the involvement of cytoskeletal elements in the infection process (Salman et al., 2005).

Chromatin targeting of T-complex

In the nucleus, the T-DNA needs to be targeted to the chromatin before its potential integration into the host genome. To understand this process, it necessary first is to determine whether *Agrobacterium* T-DNA is integrated (and, by implication, chromatin-targeted) randomly or preferentially in specific genomic domains. A series of studies, analyzing genome-wide distribution of T-DNA integration sites in *Arabidopsis*, indicated a bias toward transcriptionally active chromatin and the regulatory regions of genes (Alonso et al., 2003; Chen et al., 2003). However, in these studies, the recovery of transgenic plants used for integration analyses relied on the expression of a reporter or a selectable marker transgene, which introduced obvious bias in the nature of the recovered insertion events: high incidence of recovery of insertions into euchromatin and underrepresentation of insertions into heterochromatic regions. However, when the integration events were recovered without selection pressure, i.e., independent on transgene expression, they proved to be truly random, without bias toward active chromatin (Dominguez et al., 2002; Kim et al., 2007). That T-DNA has access to all areas of the host chromatin raises the question of a mechanism modifying the structure of the heterochromatin to render it accessible to T-DNA during *Agrobacterium* infection. Interestingly, an earlier study suggested that T-DNA integration may require that the host cell go through the S-phase of the cell cycle (Villemont, 1997), during which chromatin decondenses. Thus, if T-DNA integration indeed, occurs when the target chromatin is already naturally decondensed, there is no need for involvement of a specific factor that induces decondensation. Alternatively, plant responses to *Agrobacterium* infection as well as to other stress factors may converge on the dedifferentiation process whereby cells first acquire stem cell-like state with decondensed chromatin prior to acquisition of a new cell fate (Grafi et al., 2011).

Some of the plant factors interacting with protein components of the T-complex may mediate its targeting to the host chromatin. For example, the VirD2 interactor CAK2M, a conserved plant ortholog of cyclin-dependent kinase-activating kinase, also binds to a subunit of RNA polymerase II, which recruits TATA box-binding proteins (TBPs) (Bako et al., 2003). The association of VirD2 with CAK2M, and with TBPs, could play a role in chromatin targeting of the T-complex, and it might also explain the bias toward T-DNA integration into gene regulatory regions found in some studies (e.g., Alonso et al., 2003). VIP1 is another potential host factor involved in chromatin targeting of the T-complex; as

a plant transcription factor (Djamei et al., 2007), it is expected to associate with the cell chromatin. Indeed, VIP1 can bind the four types of *Xenopus* core histones *in vitro* (Loyer et al., 2005), and at least one plant histone, H2A, *in vivo* (Li et al., 2005a). Moreover, VIP1 exhibits strong interaction with purified plant nucleosomes *in vitro* (Lacroix et al., 2008); because this interaction is competitively inhibited by H2A, this histone likely represents a VIP1 binding site in a nucleosome context. Furthermore, VIP1 is also able to mediate the association of VirE2 as well as the synthetic minimal T-complex to plant nucleosomes *in vitro* by formation of the quaternary nucleosome-VIP1-VirE2-ssDNA complexes (Lacroix et al., 2008). This model of VIP1-mediated T-complex chromatin targeting is also consistent with the requirement for H2A and other core histones for T-DNA integration (Mysore et al., 2000; Yi et al., 2002).

Like VIP1, VIP2 is a plant transcription factor that interacts with VirE2 (Anand et al., 2007). Because VIP2 is required for stable plant genetic transformation, but not for transient T-DNA expression, it is thought to play a role in T-DNA integration (Anand et al., 2007). The mechanism of the VIP2 effect on T-DNA integration is not yet understood. As a transcription factor, VIP2 modifies the expression levels of many genes, including core histones (Anand et al., 2007), suggesting that it may affect T-DNA integration indirectly via altering histone expression. Or, VIP2, also like VIP1, could be involved in chromatin targeting, mediating between VirE2 of the T-complex and chromatin.

Potential role of other host factors in the T-complex chromatin targeting is also likely. For example, because double-stranded DNA break (DSB) repair is the main mechanism involved in T-DNA integration (Chilton and Que, 2003; Salomon and Puchta, 1998; Tzfira et al., 2003) (see below), interaction between the T-complex and a component of the host DNA repair machinery and/or DSB-associated proteins—such as BRCA which specifically localizes to DSBs, or a phosphorylated form of histone H2AX (γ H2AX) which delineates chromatin domain around DSB (Friesner et al., 2005)—might help direct the T-complex to DSBs in the host chromatin.

Disassembly of T-complex

Whereas VirE2 and VIP1 are critical for nuclear import and chromatin targeting of the T-complex, they become a liability for integration as they physically mask the DNA molecule. Thus, once the T-complex reaches the host chromatin, its proteins must be removed for the reactions of the second strand synthesis (see below) and integration. This disassembly is mediated by the host ubiquitin/proteasome system (UPS) (Tzfira et al., 2004b; Zaltsman et al., 2010a; Zaltsman et al., 2010b). The first indication for the involvement of UPS in *Agrobacterium* infection came from identification of VirF, a bacterial host range factor exported into the host cell (Regensburg-Tuink and Hooykaas, 1993), as an F-box protein (Schrammeijer et al., 2001) and subsequent identification of its cellular substrate, VIP1 (Tzfira et al., 2004b). Characteristic of the F-box proteins (Ho et al., 2006; Lechner et al., 2006), VirF interacts with several *Arabidopsis* ASK proteins (Schrammeijer et al., 2001), homologs of the yeast Skp1 component of SCF. F-box proteins and Skp1 are components of the SCF (Skp1-Cullin-F-box protein) complexes, which represent E3 ubiquitin ligases

mediating targeted protein destabilization by the 26S proteasome (Cardozo and Pagano, 2004).

Both VirF and ASK1 are located in the plant cell nucleus (Schrammeijer et al., 2001; Tzfira et al., 2004b), where the T-complex uncoating is expected to occur. VirF binds to VIP1 and promotes its degradation in plant and yeast cells (Tzfira et al., 2004b). Moreover, VirF is able to promote also the degradation of VirE2 in the presence of VIP1, even though VirF does not recognize VirE2 directly, suggesting that VirF can induce the destabilization of the entire VIP1-VirE2 complex. VIP1 and VirE2 destabilization occurs via the SCF^{VirF} pathway; indeed, in yeast cells, this destabilization does not occur in a conditional *skp1-4* mutant and requires the presence of active Skp1 (Tzfira et al., 2004b). An additional layer of regulation of the SCF^{VirF} activity may be introduced by the bacterial exported effector VirD5, which binds VirF and prevents its rapid turnover by the defensive action of the host UPS (Magori and Citovsky, 2011).

VirF, considered as a host range factor, enhances *Agrobacterium* infectivity in tomato and *Nicotiana glauca*, but is not required for infection of tobacco or *Arabidopsis* plants (Melchers et al., 1990; Regensburg-Tuink and Hooykaas, 1993). Potentially, the plant species that do not require VirF, possess an endogenous F-box protein able to fulfill the VirF function during *Agrobacterium* infection. Indeed, a VIP1-binding F-box protein (VBF), an *Arabidopsis* F-box protein induced by *Agrobacterium* infection (Ditt et al., 2006), was identified and found to bind VIP1 and promote proteasomal destabilization via the SCF^{VBF} pathway of VIP1 and VIP1-VirE2 complexes in yeast and plant cells (Zaltsman et al., 2010b). In addition, an *Arabidopsis* mutant impaired in VBF expression displayed increased resistance to *Agrobacterium*-induced tumor formation, whereas VBF expressed in *Agrobacterium* and exported to the host cell enhanced infectivity of a VirF-lacking bacterial strain in tomato plants (Zaltsman et al., 2010b). The ability of VBF to promote unmasking of ssDNA packaged by VirE2 was demonstrated directly in a cell-free system, where uncoating of synthetic minimal T-complexes and exposure of ssDNA by plant protein extracts was accelerated in presence of exogenous VIP1 and inhibited by proteasome-specific inhibitors, and was dependent of the presence of a functional VBF (Zaltsman et al., 2013).

Integration of Transferred-DNA

Recent discoveries have substantially changed our vision of the mechanism of *Agrobacterium* T-DNA integration into the host genome (Tzfira et al., 2004a). Generally, studies of T-DNA integration in plant, in yeast, and *in vitro* experimental systems have demonstrated that integration is largely dependent on the host DNA repair machinery and relegated comparatively minor roles in the integration process to bacterial T-DNA-associated proteins, which likely function as molecular links between the T-DNA and host factors. For example, initially VirD2 was thought to act as an integrase or a ligase (Pansegrau et al., 1993; Tinland et al., 1995). However, later studies have shown that host factors, but not VirD2, mediate T-DNA ligation in an *in vitro* system (Ziemienowicz et al., 2000), whereas any role that VirD2 might play in integration would be by recruiting these host factors.

Additional evidence for the involvement of cellular factors in T-DNA integration derives from the use of *Saccharomyces cerevisiae* as a heterologous host for *Agrobacterium* (Bundock et al., 1995). In yeast, foreign DNA integration can occur either by non-homologous end joining (NHEJ) or by homologous recombination (HR), depending on the presence in the integrating DNA of sequences homologous to a target sequence in the yeast genome. By using yeast mutants impaired in either in HR or in NHEJ machinery, it is possible to direct T-DNA integration toward one of those pathways (Van Attikum et al., 2001; Van Attikum and Hooykaas, 2003). The involvement of specific host proteins in each of these T-DNA integration pathways was demonstrated. For HR, two host proteins were required: Rad52, an ssDNA-binding protein, and Rad51 involved in homologous DNA pairing and strand exchange reaction (Van Attikum and Hooykaas, 2003). For NHEJ, the following host proteins were necessary: Ku70, a double-stranded DNA-binding protein that functions in heterodimer with Ku80, and Mre11, which functions in complex with Rad50 and Xrs2 and has an exonuclease activity, Sir4, and Lig4, a DNA ligase (Van Attikum et al., 2001). In a double mutant disrupted in *Rad52* and *Ku70*, the two key genes in the HR and NHEJ pathways, respectively, no T-DNA integration at all was observed (Van Attikum and Hooykaas, 2003).

How the requirements for T-DNA integration in yeast compare to integration in plants, the natural *Agrobacterium* hosts? In higher plants, NHEJ is the main pathway of foreign DNA integration (Fig. 4), whereas HR occurs only at very low rates (Gheysen et al., 1991; Mayerhofer et al., 1991). Several studies involving plant mutants in genes of the HR and NHEJ pathways have supported their overall role in T-DNA integration, although the specific results were sometimes difficult to interpret. In *Arabidopsis*, AtLig4 and AtKu80 were reported to be required for T-DNA integration in two studies (Friesner and Britt, 2003; Li et al., 2005b), but found to be dispensable for integration in another study (Gallego et al., 2003). These discrepancies might originate in the different techniques used for transformation, i.e., floral-dipping versus root tissue regeneration, or reflect more complex and redundant pathways for HR and NHEJ in plants. In addition, a mutant in the *AtRAD5* gene, closely related to the yeast *RAD51* gene involved in HR, displayed a reduced susceptibility to *Agrobacterium* infection (Sonti et al., 1995). In rice, plant lines down-regulated in *Ku70*, *Ku80*, and *Lig4* all showed strongly reduced rates of overall T-DNA integration; interestingly, the rate of HR integration was relatively increased in these three mutant rice lines (Nishizawa-Yokoi et al., 2012). The T-DNA integration pathway in plants can also be manipulated by ectopic expression of the components of the HR pathway. For example, transgenic *Arabidopsis* expressing the yeast *RAD54* displayed a significant increase in frequency of T-DNA integration by HR (Shaked et al., 2005). Intriguingly, a recent study reported that down-regulation of *XRCC4*, a major component of the NHR pathway, in *Arabidopsis* and *Nicotiana benthamiana*, resulted in increased rates of T-DNA integration, whereas the opposite effect was observed in plants overexpressing *XRCC4* (Vaghchhipawala et al., 2012). The same study also reported interaction between *XRCC4* and the *Agrobacterium* VirE2 protein, which was suggested to prevent efficient DSB repair in order to increase the probability for the T-DNA to be targeted to unrepaired DSBs (Vaghchhipawala et al., 2012).

Besides DNA repair machinery, host proteins that are mainly involved in chromatin structure or remodeling are important for T-DNA integration. Core histones, particularly H2A, are required for efficient T-DNA integration in the host genome (Mysore et al., 2000; Yi et al., 2002), which may be linked to the ability of VIP1 to bridge between histones and the T-complex during chromatin targeting (see above) (Lacroix et al., 2008; Li et al., 2005a; Loyter et al., 2005). Also, *Arabidopsis* mutants deficient in chromatin assembly factor 1 (CAF-1) were more sensitive to stable transformation by *Agrobacterium* than the wild-type plants (Endo et al., 2006); CAF-1 is involved in chromatin remodeling, and might represent a factor limiting T-DNA integration. Interestingly, the relative frequency of T-DNA integration by HR was higher in CAF-1 deficient plants (Endo et al., 2006). Finally, VIP2 is also involved in T-DNA integration, potentially by regulating histone gene transcription (see above) (Anand et al., 2007).

Another important question in regard to the mechanism(s) of T-DNA integration is whether the invading T-strand is converted to a double stranded form before or after the integration event. One of the early models for T-DNA integration, termed single-stranded gap repair (SSGR), postulated that T-DNA integration begins with annealing of the T-strand RB to the host genomic DNA via microhomologies, followed by synthesis of the second strand and ligation of T-DNA LB (Tinland, 1996). Subsequent studies have challenged this model (Tzfira et al., 2004a). The analysis of a larger number of T-DNA integration sites has revealed integration patterns incompatible with the SSGR model: microhomologies are not consistently observed at the integration sites (Alonso et al., 2003), and SSGR cannot explain some of the complex integration patterns with multiple T-DNAs in direct or reverse orientation with or without filler DNA. Specifically, the occurrence of two T-DNA molecules integrated in the head-to-head configuration is not compatible with the SSGR model because head-to-head recombination is not possible for single-stranded DNA. Similarly, the SSGR model cannot explain the presence of filler DNA (Tzfira et al., 2004a). Instead, several lines of evidence indicate that the T-strand is converted into a double-stranded form before integration. The generation of DSBs using a rare cutting endonuclease resulted in increased frequency of foreign DNA integration after *Agrobacterium*-mediated transformation (Salomon and Puchta, 1998), consistent with the higher frequency of transgene integration after X-ray treatment, known to induce DSBs (Leskov et al., 2001). These observations suggested that T-DNA likely integrates into DSBs in a double-stranded form. The direct proof of this notion was supplied a few years later by using rare cutting endonuclease sites in both the host genome and the T-DNA (Chilton and Que, 2003; Tzfira et al., 2003). Analyses of integration sites of T-DNA molecules exposed *in vivo* to a transiently expressed rare-cutting endonuclease revealed precise reconstitution after ligation of the original restriction site at the junction between the T-DNA and the host DNA, which had been also digested by the same enzyme. This is possible only if the T-strand has been converted to a double-stranded form before integration, because the endonucleases used in these studies can cleave only double-stranded DNA (Chilton and Que, 2003; Tzfira et al., 2003). Obviously, it cannot be excluded that several pathways for T-DNA integration coexist, but most of the recent data advocate for integration in a double stranded form generated from the T-strand molecule by the host DNA repair machinery. Major findings

described in this section are summarized in a simple model for T-DNA integration presented in Fig. 4.

Regulation of Transferred-DNA expression and plant resistance to *Agrobacterium*

T-DNA expression is regulated by various host factors that affect the activity of the promoters of genes naturally present within the wild type T-DNA. For example, the nopaline synthase promoter responds positively to wound auxin induction (An et al., 1990), and the promoter of the *Atu6002* gene is activated when cell division is induced, such as during the tumor growth (Lacroix and Citovsky, unpublished data). There is long-known, yet still poorly understood, variability in susceptibility to *Agrobacterium*-mediated genetic transformation not only between plant species, but also between different tissues and different physiological conditions of the host. For example, different ecotypes of *Arabidopsis* have different levels of susceptibility, and pre-treatment with growth regulators also modifies the susceptibility of a given tissue (Chateau et al., 2000). This variability likely reflects the ability of the host plant to mount a defense reaction and/or to restrict the T-DNA transfer and gene expression, as well as the ability of *Agrobacterium* to escape host defense mechanisms and/or to subvert them for enhancement of its infectivity.

Traditionally, T-DNA expression is classified into two modes: transient and stable. Transient expression is a phenomenon usually defined as a peak in T-DNA expression that occurs early, within 2-4 days, after transformation (Janssen and Gardner, 1990; Nam et al., 1999; Narasimhulu et al., 1996), and which then is decreased, both in terms of the number of expressing cells and in the expression levels per transformed cell. In contrast, late gene expression, which occurs 10-14 days after infection (Janssen and Gardner, 1990), is stable—and inheritable in the case of germline transformation (Bent, 2006)—resulting from the integrated T-DNA.

Several mechanisms likely account for the duration and levels of T-DNA expression. First, transient expression likely occurs from the T-DNA molecules that have not yet integrated into the host genome; the inherent instability of such unintegrated T-DNA would limit the duration of its expression. Indeed, T-DNA gene expression can be detected very early after inoculation, presumably before integration (Narasimhulu et al., 1996). Consistent with this notion, several plant mutants with reduced tumor formation still display normal levels of transient T-DNA expression (Li et al., 2005b; Nam et al., 1999; Zhu et al., 2003b), indicating that transient and stable expression can be uncoupled and that transient expression does not require the integration event. Furthermore, even in plant hosts that support T-DNA integration, expression of non-integrated T-DNA could be demonstrated directly by co-transformation with two T-DNAs: the C-T-DNA with the *Cre* recombinase expression cassette, and the K-T-DNA with the *nptII* expression cassette and *GUS* expression cassette flanked by two *lox* sites. These experiments generated transgenic plants that contained integrated *Cre*-processed K-T-DNA, indicating expression of the C-T-DNA, yet did not contain integrated copies of C-T-DNA (De Buck et al., 2000). Recent intriguing data suggest that T-DNA transient expression may involve not just individual unintegrated molecules, but

more complex extra-chromosomal structures composed of these molecules and generated after *Agrobacterium* infection (Singer et al., 2012), which may be involved in usually high level of transient expression.

In addition, the expression levels of integrated T-DNA are affected by different host-related factors. Mechanisms that limit expression of integrated T-DNA include death of some of the initially transformed cells (this mechanism is especially important for tumor formation or for systems involving plant regeneration from transformed cells via a callus stage), and loss of the integrated sequences due to intra-genomic reorganization. The host RNA silencing defense, however, represents the major negative regulator of T-DNA expression, both transient and stable.

RNA silencing represents a plant defense response against attack of foreign genetic material, particularly against viruses (Ding and Voinnet, 2007; Hooykaas, 1994). Silencing mechanisms are also involved in the host defense against *Agrobacterium* infection; specifically, small interfering RNAs (siRNAs) specific for the T-DNA sequence are generated by the host plant during *Agrobacterium* infection (Dunoyer et al., 2006), and plants deficient in siRNA pathways are hypersusceptible to *Agrobacterium*. Conversely, the miRNA pathway seems to be required for disease development (Dunoyer et al., 2006). This silencing response to the invading bacterial DNA likely contributes to the limited and relatively early timing of high levels of transient T-DNA expression observed in most transformations experiments: this time period may be required for the host to mount the defense reaction, after which RNA silencing takes effect, leading to reduction in T-DNA gene expression. Supporting this idea, expression of RNA silencing suppressors encoded by diverse plant viruses, such as P19 of *Tomato bushy stunt virus*, HcPro of *Potato virus Y*, or V2 of *Tomato yellow leaf curl virus*, during *Agrobacterium* infection, significantly increased the level and duration of transient T-DNA expression (Voinnet et al., 2003; Zracha et al., 2007). Although, by analogy to plant viruses most of which encode silencing suppressors (Qu and Morris, 2005), evolution of an RNA silencing suppressor in *Agrobacterium* would make biological sense, such suppressor has not been identified to date. While a decrease of siRNAs corresponding to the T-DNA sequences levels was observed in developing tumors, it was attributed to modifications in hormonal status of tumor tissues, rather than to a putative *Agrobacterium* silencing suppressor (Dunoyer et al., 2006).

Another mechanism regulating T-DNA expression may be DNA methylation, which is often observed in integrated transgenes, particularly when several T-DNA copies are integrated in the host genome (Gelvin et al., 1983; Hepburn et al., 1983). A recent study described genome-wide changes in the extent of DNA methylation in crown gall tumors as compared to mock-inoculated tissues (Gohlke et al., 2013). However, these changes may be not directly linked to *Agrobacterium* infection and/or genetic transformation, but represent indirect effects of global changes in the activity of plant growth regulators that induce cell division and tumor formation. Indeed, directing plant cell cultures to different developmental pathways by addition of plant growth regulators results in major modifications of DNA methylation as well as in other epigenetic modifications (Miguel and Marum, 2011).

Although *Agrobacterium* usually does not trigger extensive visible symptoms of plant hypersensitive defense reaction, such as necrosis, studies of changes in the host transcriptional activity induced by *Agrobacterium* infection have shown that related defense reaction mechanisms are activated. In *Arabidopsis* cells, many genes known to be involved in plant defense response are activated 48 hours after inoculation with *Agrobacterium* as compared to mock-inoculated cells (Ditt et al., 2006). A kinetic study of expression of several of these genes during the infection process revealed that the initial induction was followed by a decrease in expression levels (Veena et al., 2003), suggesting that these defense genes may be suppressed by as yet unknown *Agrobacterium* factors. In addition, an *Arabidopsis* mutant in the *EFR* gene, encoding a cell surface receptor that activates the basal defense pathway in response to pathogen-associated molecular patterns (PAMPs), such as EF-Tu, displayed hyper-susceptibility to *Agrobacterium* (Zipfel et al., 2006). Thus, the ability of *Arabidopsis* to detect bacterial pathogens and trigger the basal defense response pathway confers a measure of resistance to *Agrobacterium* infection. Production of salicylic acid (SA), the hallmark of the systemic acquired resistance (SAR) pathway, by the infected plant has also been shown to attenuate its susceptibility to *Agrobacterium* (Anand et al., 2008; Yuan et al., 2007). This ability of *Agrobacterium* to induce SAR, however, may depend on the host species, tissues, or the inoculation method. For example, *Agrobacterium* co-incubated with *Arabidopsis* seedlings modulated SAR by reducing SA accumulation and transcript levels of pathogenesis-related genes *PR-1* and *PR-5* (Gaspar et al., 2004). Predictably, this repression of SAR by *Agrobacterium* requires bacterial attachment to the host cell; when the attachment was compromised in a *rat1 Arabidopsis* mutant defective for the lysine-rich arabinogalactan protein, AtAGP17, the plants became resistant to *Agrobacterium* and did not display reduced expression of *PR-1* and *PR-5* upon infection (Gaspar et al., 2004). Unlike *Arabidopsis*, tobacco plants inoculated with *Agrobacterium* by leaf infiltration exhibited increased expression of the *PR-1* gene (Pruss et al., 2008), at levels sufficient to elicit resistance to *Tobacco mosaic virus* (TMV). This induction of *PR-1* expression was not dependent on the presence of the Ti-plasmid, likely representing a non-specific host response to bacterial challenge. The micro-RNA miR393, that represses auxin signaling and promotes antibacterial resistance, was also elevated following *Agrobacterium* infection of tobacco (Pruss et al., 2008). However, unlike *PR-1*, miR393 was induced only by *Agrobacterium* harboring a Ti-plasmid, suggesting a host cell reaction to the transfer of foreign genetic material and/or proteins.

“Arms race” in *Agrobacterium*-plant interactions

Host-pathogen interactions often represent an “arms race”, in which the host attempts to ward off invaders while the pathogen strives to suppress the host’s defense and even subvert it for the benefit of infection. This strategy of taking advantage of the host defense/stress response pathways is employed by *Agrobacterium* for genetic transformation of plant cells. First, *Agrobacterium* utilizes plant phenolics—a class of chemical compounds which includes antibacterial substances, phytoalexins, normally produced during defense response—as signals that induce the bacterial *vir* genes and activate the virulence system. *Agrobacterium* also subverts a defensive host MAP kinase signaling pathway to facilitate nuclear import and chromatin targeting of its T-complexes. Specifically, the host plant

responds to *Agrobacterium* infection by inducing MPK3 that directly phosphorylates VIP1 and activates its nuclear import (Djamei et al., 2007), which in turn facilitates nuclear import of the T-complex (Tzfira et al., 2001), whereas nuclear VIP1 then facilitates intranuclear transport of the T-complex to the target chromatin (Lacroix et al., 2008). Interestingly, an *Arabidopsis* mutant in basal immune response inhibitor *WRKY17* displays increased levels of *VIP1* transcription and is more susceptible to *Agrobacterium* than the wild-type plant (Lacroix, 2013b). Further, *Agrobacterium* infection also induces expression of the host F-box protein VBF which it is thought to utilize for proteasomal disassembly of the T-complex (Zaltsman et al., 2010b) and exposure of the T-strand (Zaltsman et al., 2013) before integration. Finally, *Agrobacterium* most likely integrates its T-DNA into DSBs by taking advantage of the host DNA repair machinery (Chilton and Que, 2003; Leskov et al., 2001; Salomon and Puchta, 1998; Tzfira et al., 2003; Tzfira et al., 2004a), which may also be considered a defense response of the cell to DNA damage.

Significance

Understanding the complex interplay between the bacterium and its host is crucial for future improvement of *Agrobacterium*'s uses in biotechnology. Indeed, the efficiency of transformation remains low for many economically-important plant species, and elucidating the function of host factors affecting T-DNA transfer and integration will help to improve transformation methods. Moreover, the *Agrobacterium*-host plant interaction has been a remarkably useful experimental system to help understand many basic cellular processes, such as cell-cell recognition, macromolecule transport, and DNA repair and integration.

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Abbreviations used in this paper:

ACC	1-aminocyclopropane-1-carboxylate
AS	acetosyringone
DIMBOA	2,4-dihydroxy-7-methoxy-2 <i>H</i> -1,4-benzixazin-3(4 <i>H</i>)-one
DSB	double strand break
HR	homologous recombination
IAA	indole acetic acid
MDIBOA	2-hydroxy-4,7-dimethoxybenzoxazin-3-one
NHEJ	non-homologous end joining
NLS	nuclear localization signal
rat	resistant to <i>Agrobacterium tumefaciens</i>

SA	salicylic acid
SCF	Skp1-Cullin-F-box protein
SAR	systemic acquired resistance
ss	single stranded
SSGR	single-strand gap repair
T-DNA	transferred DNA
Ti-plasmid	Tumor-inducing plasmid
T4SS	type four secretion system
UPS	ubiquitin/proteasome system
VBF	VIP1 binding F-box protein
VIP	VirE2 interacting protein
<i>vir</i> genes	virulence genes

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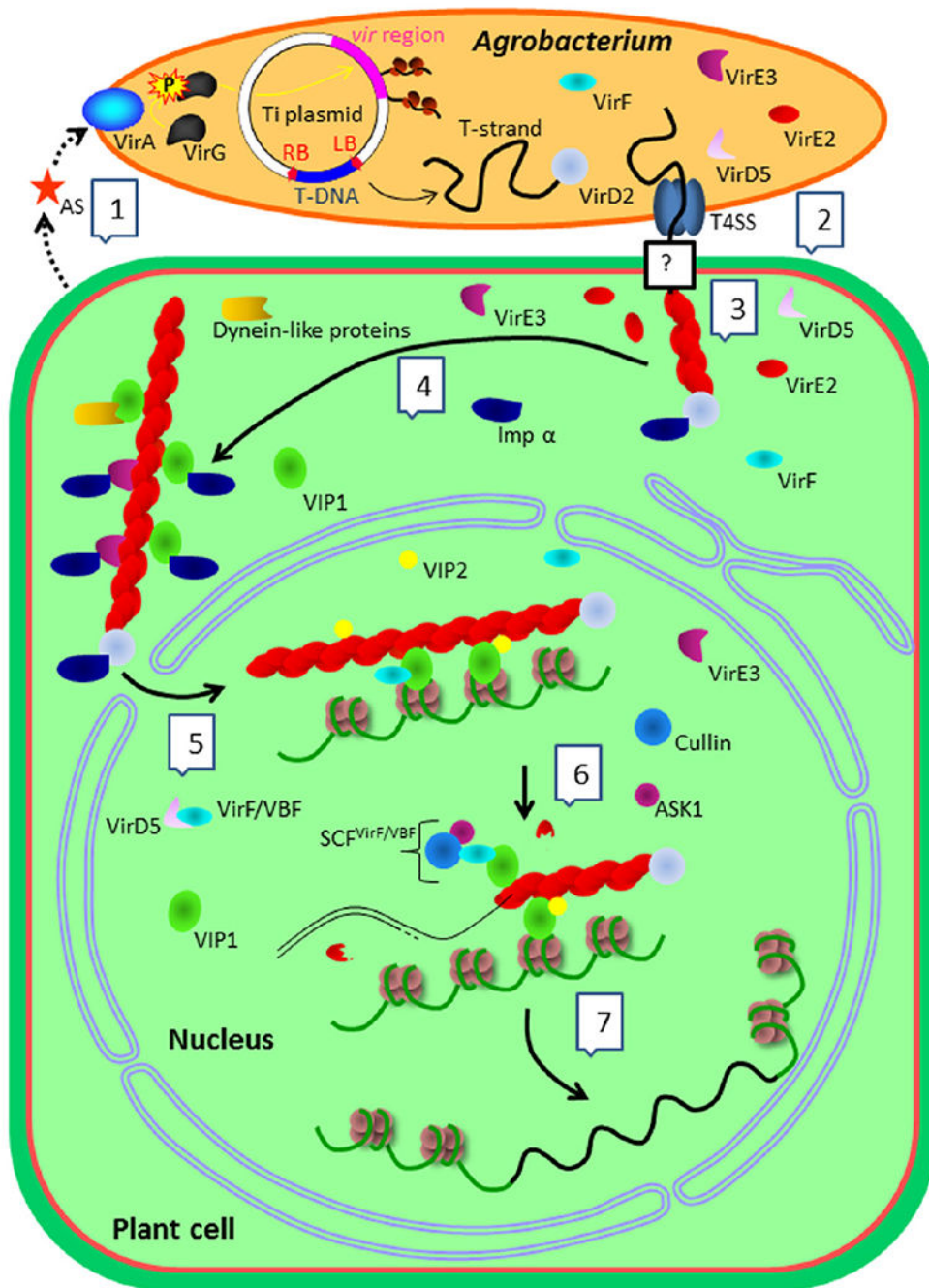


Fig. 1. Major steps in the process of transferred DNA (T-DNA) transfer and integration. Phenolic compounds, such as acetosyringone (AS), emitted by wounded plant tissue activate Agrobacterium vir gene expression via the VirA-VirG sensor (step 1), which results in generation of a mobile single-stranded T-DNA copy (T-strand). A complex composed of the T-strand and VirD2 covalently attached to its 5' end is transported to the host cell cytoplasm via the bacterial type 4 secretion system (T4SS) (step 2), which also transports into the host cell four other bacterial virulence effectors (VirD5, VirE2, VirE3, and VirF). In the host cytoplasm, the mature T-complex is assembled by cooperative binding of VirE2 molecules

along the T-strand molecule (step 3), and it is directed into the nucleus via interactions with the host cell proteins such as importin α , VIP1 (or the bacterial VirE3), and dynein-like proteins, such as DLC3 (step 4). In the nucleus, the T-complex is targeted, presumably by interactions between VIP1 and the host chromatin, to the integration site (step 5), the associated proteins are removed by proteasomal degradation via the SCF^{VirF/VBF} pathway mediated by VirF or its host functional analog VBF (step 6), The T-strand is converted to a double-stranded form and integrated into the host genome by the host DNA repair machinery (step 7).

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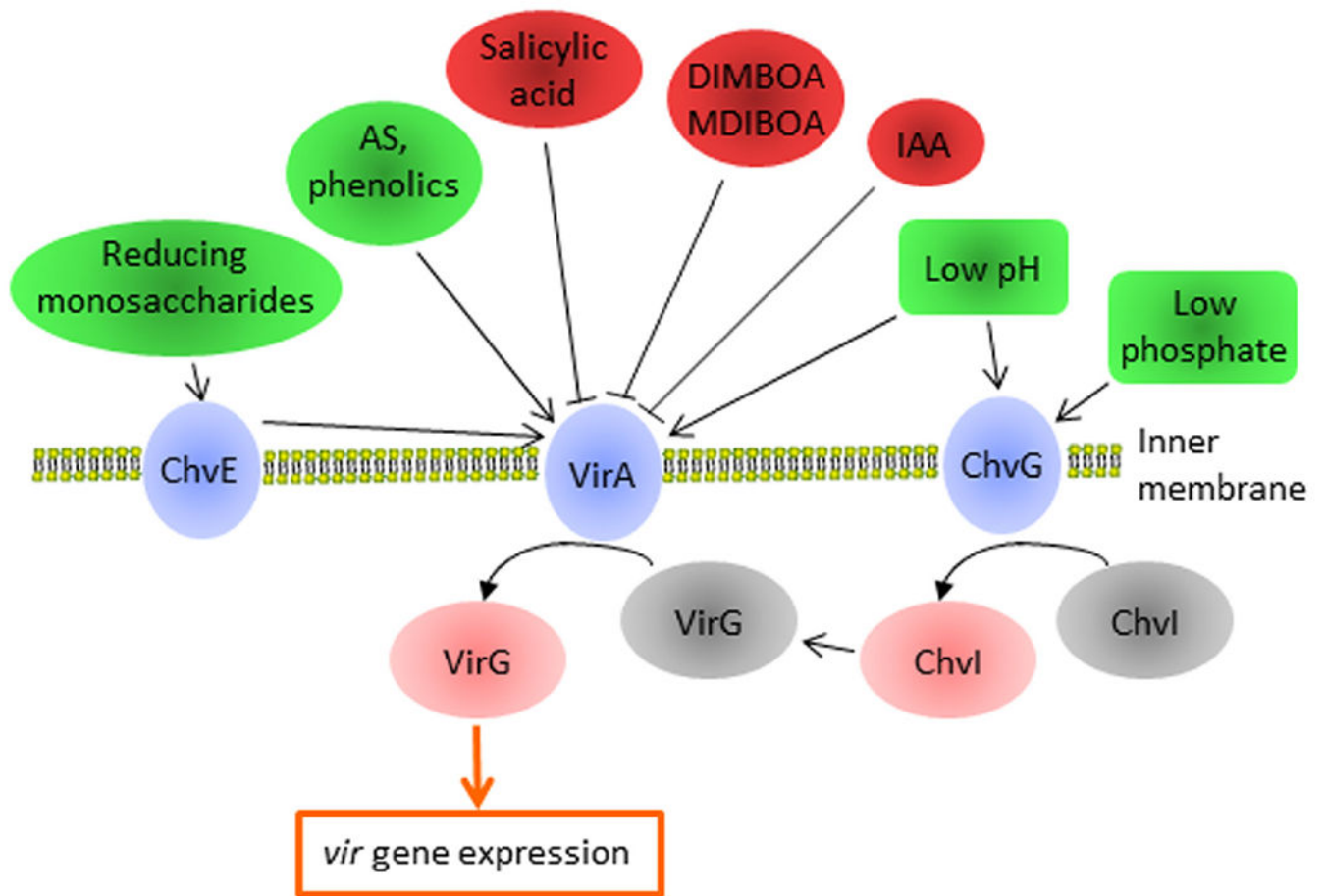


Fig. 2. Plant factors affecting *vir* gene expression.

The *Agrobacterium* VirA/VirG two-component regulatory system integrates numerous plant and environmental signals to regulate transcription of the *vir* genes. Small molecules directly bind VirA to promote [acetosyringone (AS) and related phenolic compounds], or inhibit (salicylic acid, DIMBOA, MDIBOA, IAA) VirG activation and enhancement of its expression. Reducing monosaccharides bind to ChvE, which in turn interacts with VirA to enhance AS-induced *vir* activation. Low pH and low phosphate concentration have a positive effect on *vir* activation by affecting either directly VirA or the ChvG-ChvI two-component system that in turn activates the *virG* expression.

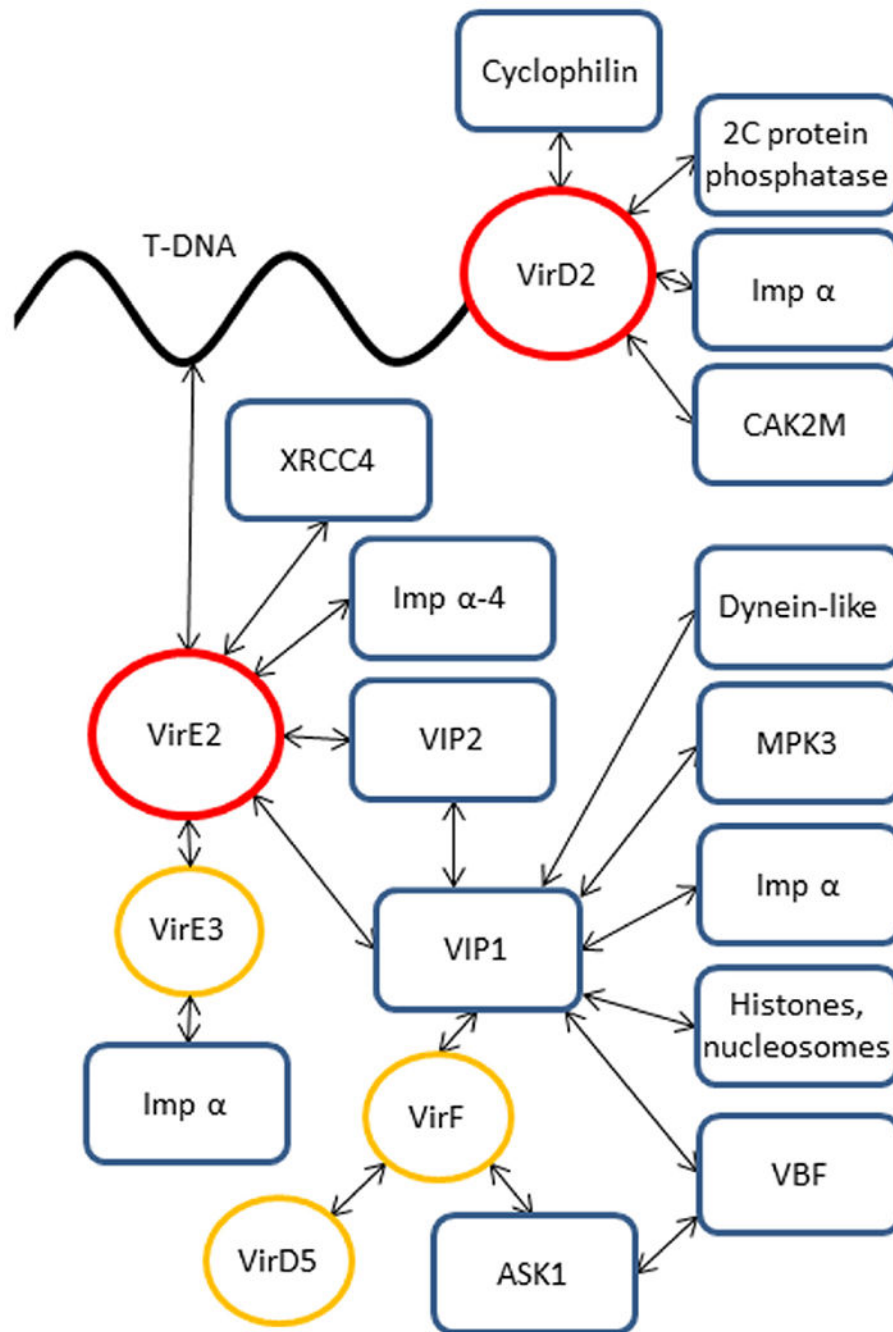


Fig. 3. Network of interactions between translocated *Agrobacterium* effectors and host cell proteins.

Blue rectangles, host factors; yellow circles, bacterial effector proteins; red circles, bacterial effector proteins directly associated with the T-strand. For other details, see text.

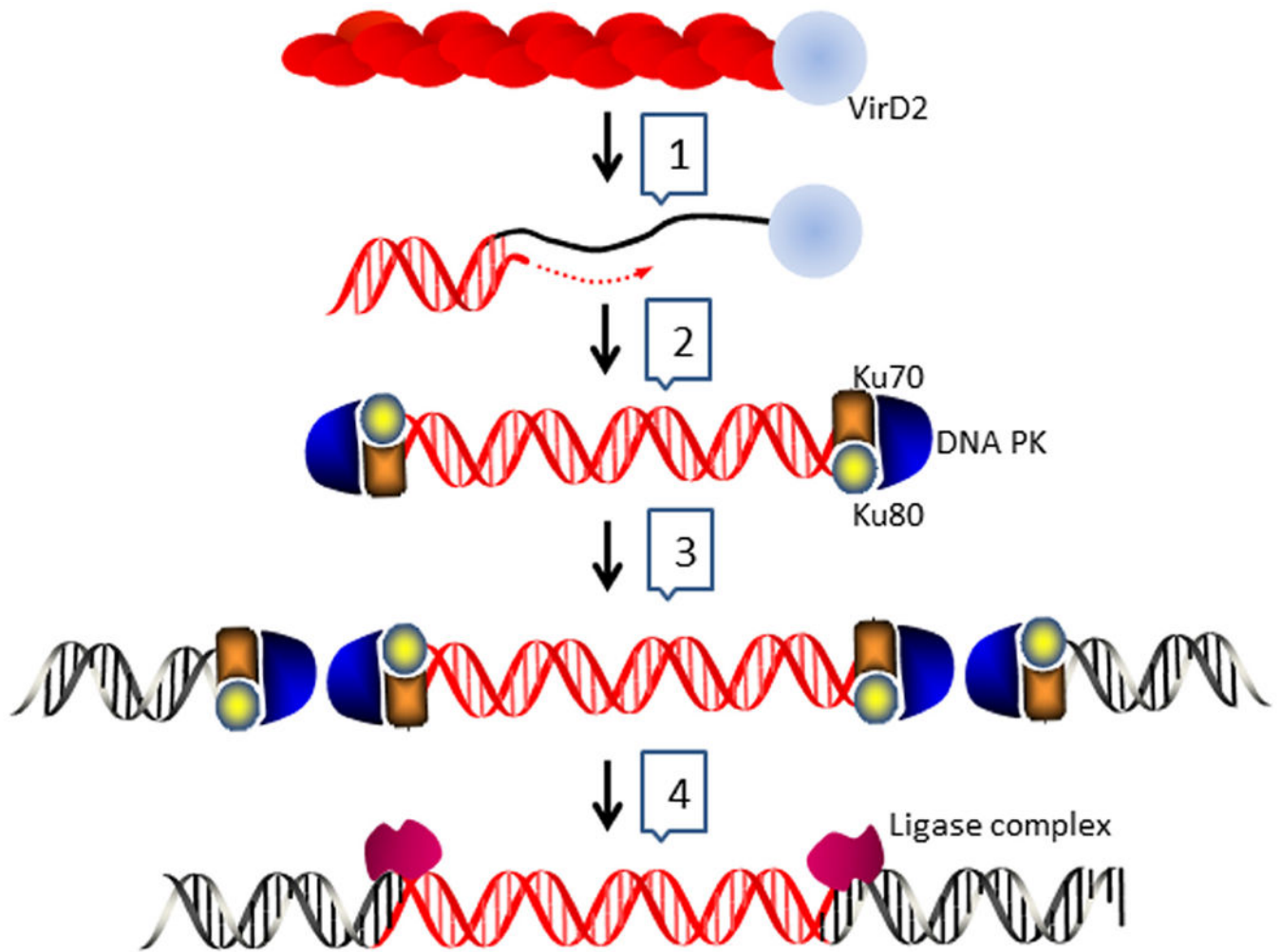


Fig. 4. Model of transferred DNA (T-DNA) integration in host cell chromatin. The T-complex is uncoated from its associated proteins (1), and converted to a double-stranded form that associates with host DNA repair machinery components, such as DNA PK, Ku70 and Ku80 (2). It then interacts with a double strand break (DSB) in the host DNA (3), and is integrated into the host genome by a host ligase activity (4).