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Microreview

Biological systems of the host cell involved in *Agrobacterium* infection

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Summary

Genetic transformation of plants by *Agrobacterium*, which in nature causes neoplastic growths, represents the only known case of *trans*-kingdom DNA transfer. Furthermore, under laboratory conditions, *Agrobacterium* can also transform a wide range of other eukaryotic species, from fungi to sea urchins to human cells. How can the *Agrobacterium* virulence machinery function in such a variety of evolutionarily distant and diverse species? The answer to this question lies in the ability of *Agrobacterium* to hijack fundamental cellular processes which are shared by most eukaryotic organisms. Our knowledge of these host cellular functions is critical for understanding the molecular mechanisms that underlie genetic transformation of eukaryotic cells. This review outlines the bacterial virulence machinery and provides a detailed discussion of seven major biological systems of the host cell–cell surface receptor arrays, cellular motors, nuclear import, chromatin targeting, targeted proteolysis, DNA repair, and plant immunity – thought to participate in the *Agrobacterium*-mediated genetic transformation.

Introduction

The ability of *Agrobacterium* to genetically transform a wide variety of plant species has earned it a place of honour in basic plant research and modern plant biotechnology. The transformation results from the production of a single-stranded copy (T-strand) of transferred DNA (T-DNA) molecule by the bacterial virulence machinery, its transfer into the host cell followed by integration into the host genome (for recent reviews, see Gelvin, 2003; McCullen and Binns, 2006). While wild-type *Agrobacterium* species are known as the causative agents of the 'crown gall' disease in a rather limited number of economically important plant species (e.g. Burr *et al*., 1998), recombinant *Agrobacterium* strains are the tool-of-choice for production of genetically modified plants in a very broad range of species (Gelvin, 2003). Furthermore, *Agrobacterium*, at least under laboratory conditions, can transform other eukaryotic species, ranging from fungi to human cells (reviewed in Lacroix *et al*., 2006a), which holds great promise for the future of biotechnology of non-plant species. This remarkably wide host range of *Agrobacterium*, which is in contrast to the relatively narrow host range of many other bacterial pathogens that are typically limited to specific species or genera – raises a question of how the *Agrobacterium* virulence machinery can function in evolutionarily distant and diverse species, crossing the interkingdom boundaries. The answer most likely lies in the ability of *Agrobacterium* to hijack fundamental cellular processes which are shared by organisms of different kingdoms. Thus, the *Agrobacterium*-mediated genetic transformation process relies both on the activity of the bacterial virulence proteins which are required for the early stages of the transformation process (e.g. host recognition and attachment, and T-strand production, Fig. 1), and on the activity of diverse host cellular proteins and systems which are required in the later stages of the transformation process (e.g. nuclear import, integration and expression of the T-DNA, Fig. 1). Here, we summarize the bacterial virulence machinery and then discuss in detail seven major biological systems of the host cell that have been implicated in the *Agrobacterium*-mediated genetic transformation. Our knowledge of these basic cellular functions, which is critical for understanding the molecular mechanisms that underlie genetic transformation of eukaryotic cells, is enhanced using *Agrobacterium* as a unique and powerful experimental tool.

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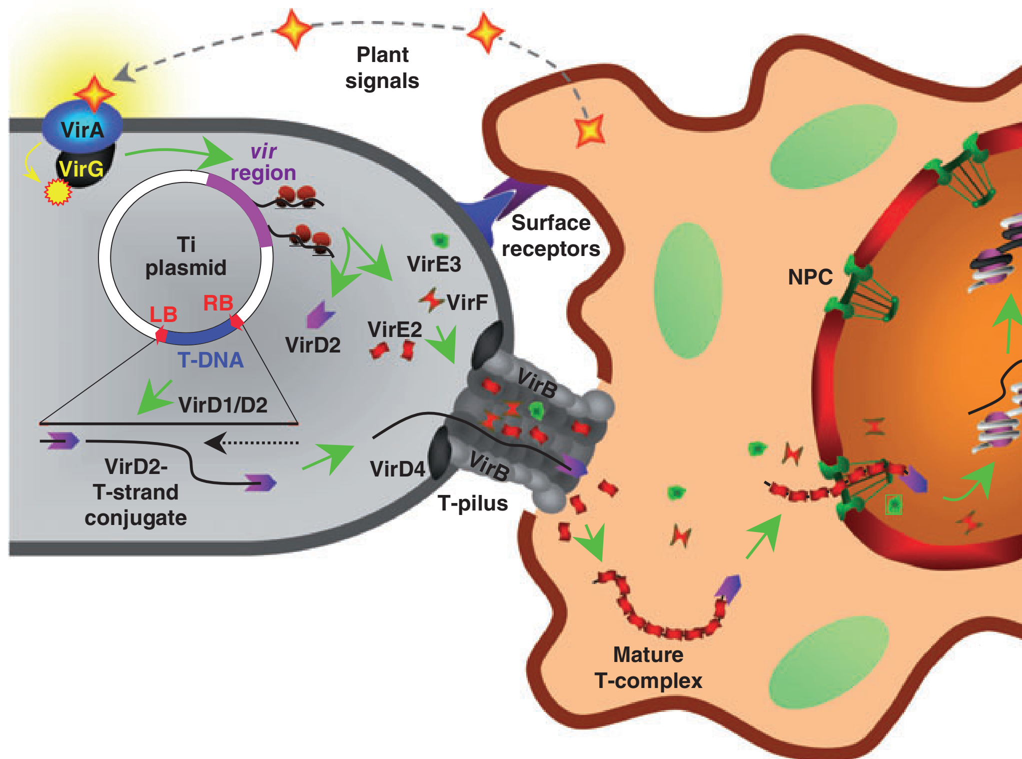

Fig. 1. Summary of major molecular events and structures within the *Agrobacterium* cell that generate the Vir protein machinery and T-strands which then are transported into the plant cell, enter its nucleus and integrate into the genome. The transformation process begins with recognition of plant signals by the bacterial VirA/VirG sensory system, followed by activation of the *vir* loci and attachment of the bacterium to the host cell. The T-strand is excised from the T-DNA region by VirD2/VirD1 and exported, *in cis* with a covalently attached VirD2 molecule and *in trans* with several other Vir proteins, into the plant cell cytoplasm via a VirB/D4 type IV secretion system. Inside the host cell, the VirD2–T-strand conjugate is packaged by numerous molecules of VirE2 to form a mature T-complex. For in-depth discussion on the T-complex transport and nuclear import, and T-DNA integration, see text.

The bacterial virulence machinery of genetic transformation: a brief overview

The *Agrobacterium*-mediated genetic transformation is a multistep process which begins with recognition and sensing of a wounded host cell by a virulent *Agrobacterium* and ends with the expression of the *Agrobacterium*'s T-DNA integrated in the transformed cell's genome. *Agrobacterium* deploys a large number of proteins and uses several molecular machines to initiate and execute the early steps of the transformation process, as illustrated in Fig. 1 and as has been previously reviewed (e.g. Gelvin, 2003; Christie *et al.*, 2005; McCullen and Binns, 2006). Briefly, proteins encoded by the bacterial chromosomal virulence (*chv*) and tumour-inducing plasmid virulence genes (*vir*) mediate recognition of and attachment to the host cell, production of a mobile T-strand-protein complex (T-complex) and its export into the host cell (Fig. 1). Once inside the host cell cytoplasm, several Vir proteins and host factors (see below) act together to deliver the T-complex into the host cell nucleus and integrate it into the host cell genome. For detailed discussion of the roles of the Vir proteins in the transformation process, the reader is referred to the recent reviews (e.g. Gelvin, 2003; Christie *et al.*, 2005; McCullen and Binns, 2006).

Plant cell surface receptors and *Agrobacterium* attachment

While *Agrobacterium* attachment to the host cell is an absolute prerequisite for transformation (reviewed in McCullen and Binns, 2006), very little is known about the nature and function of the factors that *Agrobacterium* utilizes as specific receptors on the host cell surface and/or cell wall. Among these putative receptors and host proteins (Fig. 2) are a vitronectin-like protein (Wagner and Matthysse, 1992), a rhicadhesin-binding protein (Swart *et al.*, 1994), a cellulose synthase-like gene (Zhu *et al.*, 2003a) and several VirB2-interacting proteins (Hwang and Gelvin, 2004). Vitronectins are family of proteins utilized as specific receptors by different pathogenic bacteria in mammalian cells (e.g. Paulsson and Wadstrom, 1990).

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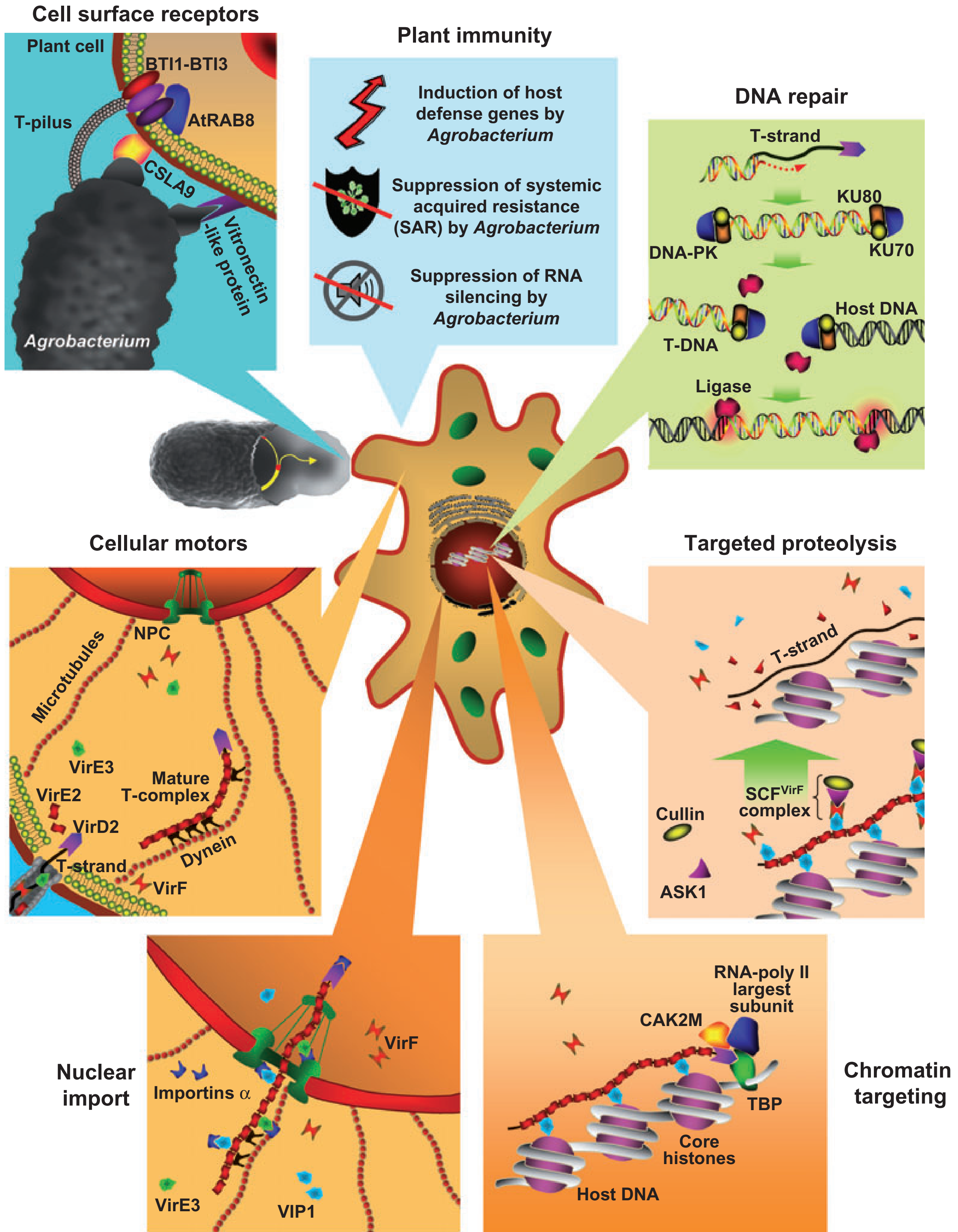

Fig. 2. Summary of major biological systems of the host cell that are involved in the *Agrobacterium*-mediate genetic transformation. Main molecular events associated with each biological system are depicted. For further details, see text.

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Because attachment of *Agrobacterium* cells to plant tissues could be inhibited by human vitronectin or anti-vitronectin antibodies and because *Agrobacterium* mutants, which are defective in their attachment ability to plant cells, showed reduced binding to vitronectin, plant vitronectin-like molecules have been suggested to play a role in *Agrobacterium* attachment to its host cells (Wagner and Matthysse, 1992); to date, however, no additional progress has been made in the studies of this putative vitronectin-like plant receptor for *Agrobacterium*.

In addition to vitronectin, another cell attachment-related factor has been postulated to participate in *Agrobacterium* binding to plant cells. This glycoprotein, isolated from cell walls of pea roots, acted as a receptor for rhicadhesin, an adhesion protein encoded by *Agrobacterium* and a related phytobacterium *Rhizobium*; similar to many cell adhesion proteins, including vitronectin, the rhicadhesin receptor contained a conserved arginine-glycine-aspartic acid cell attachment motif (Swart *et al.*, 1994).

Another host factor, a cellulose synthase-like protein CSLA9, potentially involved in *Agrobacterium* attachment, was identified by screening *Arabidopsis* mutants for resistance to *Agrobacterium* transformation. Disruption of the *CSLA9* gene in *Arabidopsis* plants resulted in a limited reduction in *Agrobacterium* attachment to inoculated roots (Zhu *et al.*, 2003a). The *CSLA9* promoter driving a *GUS* reporter gene was active in the root elongation zone (Zhu *et al.*, 2003a), an area previously shown to be most susceptible to *Agrobacterium* infection (Yi *et al.*, 2002). Thus, while the precise function of the CSLA9 protein in the transformation process remains unknown, its expression pattern – as well as that of H2A, an *Arabidopsis* core histone involved T-DNA integration (see below and Yi *et al.*, 2002) – suggests that *Agrobacterium* preferentially infects host cells and tissues at specific developmental stages. This notion is lent additional support by the observations that entry into the S-phase of the cell cycle was absolutely essential for the *Agrobacterium*-mediated stable genetic transformation of synchronized petunia cell populations (Villemont *et al.*, 1997).

Finally, several *Arabidopsis* proteins have been identified (Hwang and Gelvin, 2004) that interact with VirB2, a major component of the bacterium-host cell attachment structure termed T-pilus (Fig. 2) (Lai and Kado, 2000). These proteins include VirB2 interactors (BTIs) BTI1, BTI2 and BTI3 with unknown functions, and a membrane-associated GTPase, AtRAB8 (Hwang and Gelvin, 2004). BTI1 and AtRAB8 were implicated in the transformation process using antisense and RNA interference transgenic plants which showed reduced susceptibility to *Agrobacterium* infection. Interestingly, BTI expression was transiently increased after *Agrobacterium* infection (Hwang and Gelvin, 2004), indicating a positive feedback communication between *Agrobacterium* and its host cell. Limited understanding of the T-pilus role in the transformation process (Kado, 2000), combined with the complete lack of knowledge of the biological function(s) of the BTI proteins, hinder our ability to develop a mechanical or molecular model for participation of these host cellular factors in the transformation process.

Molecular motors and intracellular movement of the T-complex

Following its entry in the host cell cytoplasm, the VirD2–T-strand conjugate most likely is coated by VirE2, forming the T-complex (reviewed in Lacroix *et al.*, 2006b), which must then travel through the cytoplasm to reach the host cell nucleus. The dense structure of the cytoplasm, which is composed of a mesh of cytoskeletal networks and greatly restricts the Brownian diffusion of macromolecules (Luby-Phelps, 2000), and the very large size of the T-complex (Abu-Arish *et al.*, 2004) suggest an active mechanism for the intracellular transport of the T-complex (Tzfira, 2006). Studies in mammalian cells have shown that many DNA viruses use dynein motor proteins and the host microtubule network as a track system for their cytoplasmic transport towards the nucleus (reviewed in Henry *et al.*, 2006), and the possibility that *Agrobacterium* has evolved to utilize the similar transport machinery of the host cell for delivery of the T-complex (Fig. 2) is intriguing. Indeed, a recent biophysical study demonstrated active transport of artificial T-complexes along microtubules in a cell-free system (Salman *et al.*, 2005).

This study used single-particle-tracking methods to track movement of fluorescently labelled VirE2–ssDNA (single-stranded DNA) complexes on a reconstituted cytoskeletal network that contained microtubules, F-actin and associated motor proteins from the *Xenopus* egg extract (Salman *et al.*, 2005). As native VirE2 molecules are not imported into the nucleus of mammalian cells (reviewed in Lacroix *et al.*, 2006b), an 'animalized' form of VirE2 was used, in which point mutations reconstitute an active nuclear localization signal (NLS) of the widely conserved, bipartite type that is actively transported into the animal cell nucleus. Automated tracking coupled with statistical analysis revealed that 'animalized' VirE2–ssDNA complexes were actively transported along the microtubule (but not actin) network and that this movement was dynein (but not kinesin)-dependent (Salman *et al.*, 2005). Thus, the *Agrobacterium* T-complex is likely transported through the host cell cytoplasm by a cellular motor-assisted mechanism (Salman *et al.*, 2005; Tzfira, 2006). The identity of these motor proteins in plant cells, however, remains obscure. Our recent data (T. Tzfira, unpublished) suggest that a dynein-like *Arabidopsis* protein (DLC3) may be involved in the intracellular trans-

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port of the *Agrobacterium* T-complexes, through interaction with another host protein, VIP1, which mediates the recognition of VirE2 by the nuclear import machinery of the plant cell (see below). In this scenario, DLC3, a motor-like protein which associates with the plant microtubule network (T. Tzfira, unpublished) may function as a molecular link between VIP1–VirE2–T-DNA complexes and the microtubule track system. Obviously, further studies are required to examine the role of DLC3, a potential member of a new family of plant molecular motors, in the T-complex movement within plant cells and to identify additional components of plant dynein-based motors likely involved in this transport process.

Nuclear import of the T-complex

The diameter of the mature T-complex (*c.* 15 nm) (Abu-Arish *et al.*, 2004) substantially exceeds the diffusion size exclusion limit of the nuclear pore complex (NPC) (9 nm), indicating that T-complexes enter the cell nucleus by an active mechanism mediated by the nuclear import machinery of the host cell (Fig. 2). Because T-complexes are polar structures, their nuclear import is thought to occur in a polar fashion where the VirD2 molecule attached to the 5′ end of the T-strand may initiate the import process (reviewed in Sheng and Citovsky, 1996). That both VirD2 and VirE2 accumulate in the plant cell nucleus (reviewed in Lacroix *et al.*, 2006b) suggests that not only VirD2, but also VirE2 is involved in the T-complex nuclear import. This concept is supported by the observations that T-DNA expression and/or tumorigenicity is reduced in plants inoculated with *Agrobacterium* strains expressing either VirD2 with a mutated NLS (e.g. Koukolikova-Nicola *et al.*, 1993; Mysore *et al.*, 1998) or VirE2 with mutations within the protein region required for its nuclear import (Dombek and Ream, 1997). Furthermore, VirE2 alone facilitated nuclear import of fluorescently labelled ssDNA microinjected into living plant cells, and this nuclear import was blocked by a non-hydrolysable GTP analogue, a known specific inhibitor of nuclear import (Zupan *et al.*, 1996). The important role of VirE2 in the T-DNA nuclear import was also demonstrated by a complementation assay, in which a mutated *Agrobacterium* strain lacking the entire VirE2 and expressing a NLS-mutated VirD2 produced tumours on VirE2-expressing transgenic plants, but not on wild-type tobacco plants (Gelvin, 1998).

While a certain functional redundancy between VirE2 and VirD2 during the T-complex nuclear import may exist, the combined action of these Vir proteins may be required for the efficient polar translocation of the T-complex into the host nucleus (Ziemienowicz *et al.*, 2001). Thus, VirD2 and VirE2 likely possess specific functional differences which allow them to perform different, but complementary functions during nuclear import of the T-complexes. Indeed, while VirD2 was imported into the nucleus of both plant and non-plant cells (e.g. Howard *et al.*, 1992; Ziemienowicz *et al.*, 1999; Rhee *et al.*, 2000), VirE2 targeted to the nuclei of plant cells (e.g. Citovsky *et al.*, 1992; 1994), but not of *Xenopus* oocytes and *Drosophila* embryos (Guralnick *et al.*, 1996), human HeLa cells (Tzfira *et al.*, 2001), or yeast cells (Rhee *et al.*, 2000; Tzfira *et al.*, 2001), suggesting that nuclear import of VirD2 and VirE2 in plant cells may occur by different mechanisms. Interestingly, when the nuclear import of VirD2 and VirE2 was studied in permeabilized human cells, both proteins were accumulated to the cell nuclei, but only VirD2, and not VirE2, was able to import ssDNA (Ziemienowicz *et al.*, 1999). In a similar *in vitro* study using plant cell nuclei, VirD2 was sufficient for the import of short ssDNA, but only the combined function of VirE2 and VirD2 allowed the import of long ssDNA molecules (Ziemienowicz *et al.*, 2001), suggesting that while VirD2 initially directs the T-strand into the NPC, VirE2 may package it in a transferable form and assist translocation of the entire T-complex into the host cell nucleus.

If VirD2 and VirE2 employ different cellular mechanisms for their nuclear entry and perform different functions during transformation, then they are expected to utilize different subsets of cellular factors for their activities. Indeed, VirD2 and VirE2 interact with host factors that belong to distinctly different biological systems of the host cell (Ballas and Citovsky, 1997; Deng *et al.*, 1998; Tzfira *et al.*, 2001; Bakó *et al.*, 2003). For example, VirD2 was shown to interact with a set of plant cyclophilins that include Roc1, Roc2, Roc3, Roc4 and Roc5/CypA (Deng *et al.*, 1998; Bakó *et al.*, 2003). Cyclophilins are a family of conserved peptidyl-prolyl *cis-trans* isomerases (PPIases) (Tao *et al.*, 2004) which also function as molecular chaperons. Cyclosporin A, known to bind cyclophilins and block their PPIase activity, inhibited the VirD2–CypA interaction *in vitro* and blocked the *Agrobacterium* infection of *Arabidopsis* and tobacco tissues (Deng *et al.*, 1998). While the exact function of CypA and other VirD2-interacting cyclophilins in *Agrobacterium* infection remains unclear, they may act as molecular chaperons that maintain the proper conformation of VirD2 during its transport through the cytoplasm and/or into the nucleus of the host cell (Deng *et al.*, 1998).

Agrobacterium may also use a host regulatory phosphorylation/dephosphorylation machinery to control the nuclear import of VirD2 which is phosphorylated by nuclear cyclin-dependent kinase-activating kinases (CAK2Ms) from alfalfa and *Arabidopsis* (Bakó *et al.*, 2003). This phosphorylation is likely reversed by a type 2C serine/threonine protein phosphatase, designated DIG3, which interacts with the NLS-containing C-terminal

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region of VirD2 (cVirD2) (Tao *et al*., 2004). Overexpression of DIG3 in tobacco BY2 protoplasts partially inhibited nuclear import of cVirD2, which led the authors to suggest that DIG3 may negatively regulate nuclear import of T-complexes (Tao *et al*., 2004).

Directly reflecting its capacity for active nuclear import, VirD2 was found to interact with AtKAPα (Ballas and Citovsky, 1997) (Fig. 2), a member of a conserved family of importin/karyopherin α proteins, known to bind NLS and mediate nuclear import in plant and non-plant cells. The interaction of AtKAPα with VirD2 depended on the presence of the C-terminal bipartite NLS of VirD2. The ability of AtKAPα to potentiate the nuclear import of VirD2 in permeabilized yeast cells, the interaction of VirD2 with three other members of the *Arabidopsis* importin α family (Bakó *et al*., 2003), and the observation that an *Arabidopsis* mutant in one of the importin α genes is resistant to *Agrobacterium* infection (Zhu *et al*., 2003b) further support the idea that *Agrobacterium* utilizes the importin α-dependent nuclear import machinery of the host cell for nuclear uptake of the invading T-complexes.

Consistent with its inability to enter the cell nucleus in non-plant systems, VirE2 failed to interact with AtKAPα in the yeast two-hybrid assay (Ballas and Citovsky, 1997). Instead, VirE2 was found to bind the *Arabidopsis* VIP1, a novel basic-zipper (bZIP) protein (Tzfira *et al*., 2001). VIP1 formed ternary complexes with VirE2 and ssDNA *in vitro*, whereas VIP1 and VIP1-VirE2 complexes accumulated in the plant cell nucleus within living plant cells (Tzfira *et al*., 2001; 2004a; Lacroix *et al*., 2005; Li *et al*., 2005a). VIP1 also underwent nuclear import in yeast and mammalian cells, and it promoted nuclear import of VirE2 in these non-plant systems, both *in vivo* and in permeabilized human cells (Tzfira *et al*., 2001; Citovsky *et al*., 2004). Because no close homologues of VIP1 were found in non-plant databases (Tzfira *et al*., 2001), VIP1 may represent the plant-specific factor responsible for the ability of plant, but not non-plant cells to support nuclear import of VirE2.

The importance of VIP1 for the transformation process was underscored by the observations that antisense suppression of VIP1 rendered the resulting transgenic plants resistant to both transient and stable genetic transformation by *Agrobacterium* (Tzfira *et al*., 2001) whereas overexpression of VIP1 increased the susceptibility to transformation (Tzfira *et al*., 2002). Because VIP1 interacted with plant and animal importins α in the yeast two-hybrid assay (Tzfira *et al*., 2002) and *in vitro* (Citovsky *et al*., 2004), and nuclear import of VIP1 in yeast cells absolutely required the presence of importin α (Tzfira *et al*., 2002), it was suggested that *Agrobacterium* uses VIP1 as a molecular adaptor between VirE2 and the importin α-dependent nuclear import pathway of the host cell (Fig. 2). Indeed, VIP1 assembled in ternary complexes with VirE2 and importin α *in vitro* (Citovsky *et al*., 2004).

Interestingly, VirE3, an *Agrobacterium* protein exported to the host plant cells (Lacroix *et al*., 2005), mimicked the function of VIP1 in the VirE2 nuclear import by interacting with both VirE2 and a plant importin α and promoting nuclear accumulation of VirE2 in mammalian cells and in plants with suppressed expression of VIP1 (Lacroix *et al*., 2005). These observations may explain how *Agrobacterium* can genetically transform non-plant cells that lack VIP1 (e.g. yeast and human cells, reviewed in Lacroix *et al*., 2006a). Thus, although *Agrobacterium* takes advantage of basic biological systems of the host cell for infection, it also may have evolved to produce a 'backup' system of Vir proteins that are exported into the host cells and augment the cellular functions critical for infection. This idea is consistent with the role of VirE3 as one of the bacterial host range factors (Hirooka and Kado, 1986), which may compensate for the absence or very low levels of VIP1 in some plants, and it may reflect a more general ability of infectious microorganisms to encode and export protein functions, likely acquired by convergent evolution, similar to those normally provided by the host cell (Nagai and Roy, 2003).

Chromatin targeting of the T-complex

Once inside the nucleus, the T-strand must be delivered to site of its future integration in the host chromatin. While the exact sequence of events that mediate this intranuclear transport and chromatin targeting is still unknown, various plant factors and several molecular mechanisms have been implicated in these concluding steps of the transformation process. Specifically, CAK2M and TATA box-binding protein (TBP) both of which bind VirD2 (Bakó *et al*., 2003), VIP1 which binds VirE2 (Tzfira *et al*., 2001) and core histones which bind VIP1 (Li *et al*., 2005a; Loyter *et al*., 2005) may function in chromatin targeting of the T-complex. CAK2M interacts with the largest subunit of RNA polymerase II, and the latter recruits TBPs not only for transcription, but also for control of transcription-coupled DNA repair. Thus, CAK2M and TBP represent the components of the plant transcriptional and DNA repair machineries, and their interaction with VirD2 (Bakó *et al*., 2003) may target the latter and its cognate T-strand and/or the entire T-complex to the host chromatin (Fig. 2).

By analogy to many other plant bZIP proteins, VIP1 may also be a component of the cellular transcription machinery; in addition, VIP1 has been implicated in decondensation of the plant chromatin (Avivi *et al*., 2004). Thus, the association of VIP1 with VirE2 (Tzfira *et al*., 2001) may promote chromatin targeting of the T-complex (Fig. 2). Indeed, VIP1 was found to interact with all four core histones of *Xenopus in vitro* (Loyter *et al*., 2005), and

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with at least one *Arabidopsis* core histone, H2A, *in planta* (Li *et al*., 2005a; Loyter *et al*., 2005), and plant H2A is known to be essential for T-DNA integration (Mysore *et al*., 2000a). The VIP1–H2A interaction was further linked to chromatin targeting by the findings that an insertional *Arabidopsis* mutant in the *VIP1* gene produced a truncated VIP1 protein still capable of supporting nuclear import of VirE2 and transient T-DNA expression, but unable to interact with H2A and promote stable expression of the T-DNA (Li *et al*., 2005a). Finally, chromatin targeting of the T-complex may be also facilitated by another VirE2-interacting protein, VIP2 (Tzfira *et al*., 2000), with a homology to Negative on TATA-less (Not) transcription factors. Consistent with this function and similarly to the truncated VIP1 mutant (Li *et al*., 2005a), VIP2 is required for stable, but not for transient transformation of plants by *Agrobacterium* (A. Anand *et al*., unpublished). Further supporting the thought that *Agrobacterium* uses the host transcription machinery for delivery of its T-DNA to points of integration in the host chromatin (Fig. 2), high frequency of T-DNA insertion was observed within the regulatory regions of plant genes (e.g. Alonso *et al*., 2003).

Targeted proteolysis and T-DNA uncoating

At least partial uncoating of the T-DNA from its escorting proteins (Fig. 2) is necessary for exposing the T-strand to the host DNA repair machinery which will complement it to the double-stranded form and integrate the latter into the host genome. Potentially, this is achieved by the targeted proteolysis machinery of the host cell. The first indication of targeted proteolysis involvement in the transformation process came from the studies of VirF, a bacterial host range factor (e.g. Regensburg-Tuink and Hooykaas, 1993) exported into the host cell (Vergunst *et al*., 2000). VirF was shown to possess an F-box domain and interact with several members of the ASK protein family of *Arabidopsis* homologues of the yeast Skp1 protein (Schrammeijer *et al*., 2001). F-box and Skp1 represent the conserved components of E3 ubiquitin ligases called SCF (Skp1-Cullin-F-box protein) complexes that mediate and specify protein destabilization by targeted proteasomal degradation (reviewed in Ho *et al*., 2006).

A later study identified VIP1 as one of the cellular substrates for VirF and demonstrated that VirF destabilizes VIP1 and its cognate VirE2 when coexpressed in yeast cells or *in planta*, and that, in yeast, this destabilization requires the presence of Skp1 (Tzfira *et al*., 2004a). Because VirE2 represents the major protein component of the T-complex, its targeted proteolysis by the SCF^{VirF} complexes may represent a mechanism for T-DNA uncoating prior to or during its integration into the host genome (Tzfira *et al*., 2004a) (Fig. 2). Consistent with this hypothesis, both VirF and ASK1 were found to localize to the plant cell nucleus, the cellular compartment in which the T-DNA uncoating is expected to occur; furthermore, early T-DNA expression was specifically inhibited by a proteasomal inhibitor (Tzfira *et al*., 2004a). Interestingly VirF is required for transformation of some but not all plant species (e.g. Regensburg-Tuink and Hooykaas, 1993), suggesting that its function may be fulfilled by as yet unidentified nuclear-localized cellular F-box protein(s), and that *Agrobacterium* may harness the host SCF pathways for its own life cycle.

DNA repair and T-DNA integration

T-DNA integration is the last and perhaps the most host-dependent step of the transformation process (reviewed in Tzfira *et al*., 2004b). Host factors are required for complementation of the T-strand molecule to double-stranded DNA (dsDNA), for production of DNA breaks in the host genome and for ligation of the T-DNA molecule into these breaks (Fig. 2). While we are still far from complete understanding of the exact steps that lead to the integration of a single-stranded T-DNA molecule into a double-stranded host genome, recent studies have begun revealing key proteins and possible mechanisms which govern the T-DNA integration process. Early models suggested a general mechanism for T-DNA integration that begins with microannealing of the T-DNA left and right borders to the host genome, followed by production of a nick in the host genome, insertion and ligation of the T-strand molecule into the nick and its conversion to a double-stranded form by gap repair (reviewed in Tzfira *et al*., 2004b). Recent evidence, however, indicates that double-stranded breaks (DSBs) in the host genome and double-stranded T-DNA intermediates play an important role in the integration process. First, induction of DSBs at specific sites in the host DNA by a rare-cutting restriction enzyme results in frequent insertion of T-DNA molecules into these sites (Salomon and Puchta, 1998). Second, incorporating a recognition site for a rare-cutting restriction enzyme not only in the plant genome, but also within the invading T-DNA resulted in frequent insertion of the digested T-DNA molecules into DSBs; because the restriction endonuclease utilized in these experiments cleaves only dsDNA (Jasin, 1996), the T-strands must have been converted to double-stranded molecules prior to their cleavage and integration into genomic DSBs (Chilton and Que, 2003; Tzfira *et al*., 2003). It is therefore likely that, also in nature, T-DNA integration involves conversion of the T-strands into double-stranded intermediates which are then directed to naturally occurring DSBs in the host genome for integration (Fig. 2).

Yeast cells, which can be genetically transformed by *Agrobacterium*, yielded much of what we know today

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about the role of host proteins in the T-DNA integration process. *Agrobacterium* infection of yeast mutants in specific DNA repair genes allowed identification of Ku70, Rad50, Mre11, Xrs2, Lig4 and Sir4 as key proteins in T-DNA integration via non-homologous (illegitimate) recombination (NHR) pathway (van Attikum and Hooykaas, 2003), and demonstrated that Rad51 and Rad52, but not Rad50, Mre11, Xrs2, Lig4 or Ku70 are essential for T-DNA integration by homologous recombination (HR) (van Attikum *et al*., 2001). Furthermore, Ku70 (van Attikum *et al*., 2001) and Rad52 (van Attikum and Hooykaas, 2003) were found to be the key determinants for T-DNA integration via HR or NHR respectively, and double mutation of the *Ku70* and *Rad52* genes resulted in complete blockage of T-DNA integration (van Attikum and Hooykaas, 2003). Another yeast DNA repair protein, Rad54, promoted a high-frequency gene targeting in transgenic plants (Shaked *et al*., 2005).

In plant cells, T-DNA integration occurs mainly through NHR, even when the T-DNA shares high homology with the host genome, indicating that *Agrobacterium* may be exclusively using the host non-homologues end-joining (NHEJ) DNA repair machinery during the integration step. Indeed, the critical role of KU80, a key participant of NHEJ which usually functions in a complex with KU70 and DNA protein kinase (reviewed in Tzfira *et al*., 2004b; Lacroix *et al*., 2006b) (Fig. 2), during T-DNA integration in *Arabidopsis* somatic tissues was recently revealed by the observations that *Arabidopsis* insertional mutants in the *KU80* gene are defective in T-DNA integration in somatic cells, and that complexes between KU80 and double-stranded T-DNA molecules can be immunoprecipitated from *Agrobacterium*-infected plants (Li *et al*., 2005b). The role of KU80 during the transformation of germ-line cells, however, is less clear as it has been reported to be both required (Friesner and Britt, 2003) and dispensable (Gallego *et al*., 2003) for T-DNA integration. Similarly, the role of the *Arabidopsis* LIG4 ligase, another NHEJ participant (Fig. 2), in the transformation process remains controversial; LIG4 was dispensable for T-DNA integration in somatic *Arabidopsis* cells (van Attikum *et al*., 2003), but it was essential for T-DNA integration in germ-line cells (Friesner and Britt, 2003). Some of these apparently contradictory results may arise from the nature of the germ-line transformation (Ye *et al*., 1999) which was used in many of these studies and performed under relatively uncontrolled conditions. Alternatively, these controversies may reflect fundamental differences between host factors required for T-DNA integration in somatic cells (Zhu *et al*., 2003b) and in germ-line cells (Ye *et al*., 1999). Indeed, many other *Arabidopsis* mutants resistant to *Agrobacterium* somatic transformation (*rat* mutants) (Zhu *et al*., 2003b) remained susceptible to the germ-line transformation (Mysore *et al*., 2000b).

T-DNA integration may also require the participation of the host DNA-packaging proteins. For example, the *Arabidopsis* histone H2A, which displays higher expression levels in tissues more susceptible to *Agrobacterium* infection (Yi *et al*., 2002), is essential for T-DNA integration in somatic cells (Mysore *et al*., 2000a). Thus, in addition or alternatively to its described above role in directing the T-complex molecules to the integration site (Li *et al*., 2005a; Loyter *et al*., 2005), H2A may be involved in relaxing the host DNA structure (Mysore *et al*., 2000a). Indeed, also as mentioned above, genome-wide T-DNA insertional analysis revealed a bias for T-DNA integration into intergenic regions which also include promoter sequences and 5′ and 3′ untranslated regions (e.g. Alonso *et al*., 2003) that are most likely to be temporarily unpacked and may serve as ‘hot points’ of attraction for T-DNA integration. In addition, because the events of integration and chromatin targeting are temporally and spatially adjacent, at least some of the host factors involved in chromatin targeting (see above) may also facilitate integration, and vice versa.

Plant immunity and T-DNA expression

Plants perceive *Agrobacterium* and the transferred transgenes as foreign invaders and use their defence systems to battle the infection process and expression of foreign genes. Recent analyses of the response of different plant species to *Agrobacterium* infection demonstrated that, in *Ageratum* plants, a number of general defence-related genes were moderately induced; this induction was dramatically higher when the plants were challenged by an attachment-defective *Agrobacterium* mutant, suggesting that *Agrobacterium* binding to the host cell may moderate its defence response (Ditt *et al*., 2001; 2005). Similarly, the *Arabidopsis* transcriptome responded to *Agrobacterium* infection by upregulating numerous general defence genes, and this induction of defence was best observed relatively late (24–48 h) after inoculation (Ditt *et al*., 2006). In tobacco, however, *Agrobacterium* infection activated defence response genes early after inoculation (6–12 h), but suppressed them later (24–36 h) in the infection (Veena *et al*., 2003).

To date, three specific plant immunity pathways have been implicated in the host response to *Agrobacterium*: perception of bacterial pathogen-associated molecular patterns (PAMPs) which leads to production of reactive oxygen species and reinforcement of cell walls via callose deposition (reviewed in Nürnberger *et al*., 2004), systemic acquired resistance (SAR) which involves accumulation of salicylic acid (SA) and activation of pathogenesis-related (PR) genes (reviewed in Durrant and Dong, 2004), and RNA silencing which promotes cleavage/translational inhibition of the target mRNA and epigenetic modification

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of its gene (reviewed in Bisaro, 2006). Recent studies indicate that while *Agrobacterium* is susceptible to the PAMP perception defence (Zipfel *et al.*, 2006), it has evolved to counteract SAR (Gaspar *et al.*, 2004) and RNA silencing (Dunoyer *et al.*, 2006).

PAMP perception likely reduces the *Agrobacterium*-induced genetic transformation because an *Arabidopsis* mutant in the *EFR* gene, which encodes a receptor kinase essential for perception of the bacterial EF-Tu PAMP, was super-susceptible to transformation (Zipfel *et al.*, 2006). In contrast, the SAR response of the host plant is likely inhibited by *Agrobacterium* infection based on the observations that infected *Arabidopsis* plants exhibited reduction in SA accumulation and *PR1* and *PR5* gene expression levels which were even lower than their already low levels in healthy plants (Gaspar *et al.*, 2004). These *Agrobacterium* effects on SAR may involve the host lysine-rich arabinogalactan-protein AtAGP17 because a mutant in the *AtAGP17* gene (*rat1*) retained its *PR1* and *PR5* expression levels in the presence of *Agrobacterium* and became resistant to the infection (Gaspar *et al.*, 2004). Besides SAR, *Agrobacterium* has evolved to counteract the RNA silencing response of the host. Intriguingly, the interrelationship between *Agrobacterium* infection and RNA silencing is very complex because whereas the development of *Agrobacterium*-induced tumours required suppression of RNA silencing mediated by short interfering RNAs (siRNAs), it mandated a functional microRNA (miRNA)-mediated silencing because miRNA-deficient plants were almost immune to infection (Dunoyer *et al.*, 2006). Furthermore, the suppression of siRNA-mediated gene silencing occurred only within tumours, but not in the tissues at earlier stages of the transformation process or upon transformation by non-oncogenic *Agrobacterium* (Dunoyer *et al.*, 2006). The silencing suppression in tumours occurred via inhibition of siRNA synthesis, potentially due to interference with Dicer (Dunoyer *et al.*, 2006). The specific factors responsible for suppression of RNA silencing in the *Agrobacterium*-induced tumours remain unknown. By analogy to plant viruses many of which encode RNA silencing suppressors (reviewed in Bisaro, 2006), *Agrobacterium* may encode and transfer to plant its own silencing suppressor(s); because the suppression occurs only in tumours, the suppressor may be specified by the oncogenic T-DNA. Alternatively, *Agrobacterium* may suppress silencing indirectly, via developmental changes induced in the transformed cells by phytohormones. In this regard, it is noteworthy that miRNA pathways are required for cell fate determination (Bartel and Bartel, 2003) which may be involved in tumorigenesis (Dunoyer *et al.*, 2006), and that *Agrobacterium* virulence is negatively feedback-regulated by phytohormones (Liu and Nester, 2006).

Concluding remarks

To date, we have come to realize that *Agrobacterium* not only uses a complex set of its own virulence functions to genetically transform the host cells, but it also subverts many basic cellular processes for this purpose. Furthermore, *Agrobacterium* is able to actively interfere with some of these systems to escape host defence. The complexity of the eukaryotic systems utilized and/or affected by *Agrobacterium* is just beginning to emerge. Better understanding of the host biological processes involved in transformation will unravel principles that govern *Agrobacterium*–host cell interactions which result in the unique event of *trans*-kingdom gene transfer, afford novel insights into the cellular processes themselves, and help develop new strategies for efficient genetic manipulation of plant and non-plant organisms.

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