Minireview

**Agrobacterium tumefaciens** and *A. rhizogenes* use different proteins to transport bacterial DNA into the plant cell nucleus

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**Summary**

*Agrobacterium tumefaciens* and *A. rhizogenes* transport single-stranded DNA (ssDNA; T-strands) and virulence proteins into plant cells through a type IV secretion system. DNA transfer initiates when VirD2 nicks border sequences in the tumour-inducing plasmid, attaches to the 5′ end, and pilots T-strands into plant cells. *Agrobacterium tumefaciens* translocates ssDNA-binding protein VirE2 into plant cells where it targets T-strands into the nucleus. Some *A. rhizogenes* strains lack VirE2 but transfer T-strands efficiently due to the GALLS gene, which complements an *A. tumefaciens* virE2 mutant. VirE2 and full-length GALLS (GALLS-FL) contain nuclear localization sequences that target these proteins to the plant cell nucleus. VirE2 binds cooperatively to T-strands allowing it to move ssDNA without ATP hydrolysis. Unlike VirE2, GALLS-FL contains ATP-binding and helicase motifs similar to those in TraA, a strand transferase involved in conjugation. VirE2 may accumulate in the nucleus and pull T-strands into the nucleus using the force generated by cooperative DNA binding. GALLS-FL accumulates inside the nucleus where its predicted ATP-dependent strand transferase may pull T-strands into the nucleus. These different mechanisms for nuclear import of T-strands may affect the efficiency and quality of transgenic events in plant biotechnology applications.

**Introduction**

*Agrobacterium rhizogenes* causes hairy root disease in which adventitious roots proliferate from infected plant tissue. Pathogenesis results when transformed plant cells express rol (root loci) genes transferred from the root-inducing (Ri) plasmid (White *et al.*, 1985). In contrast, *A. tumefaciens* causes unorganized growth of infected plant cells. Oncogenes transferred from the tumour-inducing (Ti) plasmid into plant cells encode proteins involved in synthesis of plant growth hormones auxin (iaaM and iaaH) and cytokinin (ipt), which results in formation of crown galls (Zhu *et al.*, 2000).

Regions of the Ri and Ti plasmids that are transferred to plant cells (T-DNA) are delimited by border sequences (Wang *et al.*, 1984; Peralta and Ream, 1985). T-DNA transfer initiates when border sequences are nicked by VirD2 and VirD1 (Yanofsky *et al.*, 1986). VirD2, which contains a secretion signal (Vergunst *et al.*, 2005), attaches to the 5′ end of the nicked strand (Herrera-Estrella *et al.*, 1988; Ward and Barnes, 1988; Young and Nester, 1988) and is transported into plant cells along with attached T-strands (Stachel *et al.*, 1986). Transport requires a type IV secretion system that includes eleven virB-encoded proteins (Christie, 1997) and VirD4 (Okamoto *et al.*, 1991). VirD2 contains a nuclear localization sequence (NLS) that mediates its nuclear import through interactions with host importin α proteins (Herrera-Estrella *et al.*, 1990; Howard *et al.*, 1992; Tinland *et al.*, 1992; Rossi *et al.*, 1993; Citovsky *et al.*, 1994; Ballas and Citovsky, 1997; Bhattacharjee *et al.*, 2008), and this NLS is required for efficient transfer of T-strands (Shurvinton *et al.*, 1992; Rossi *et al.*, 1993; Narasimhulu *et al.*, 1996; Mysore *et al.*, 1998).

Tumour-inducing and root-inducing plasmids encode different effector proteins essential for gene transfer

Root-inducing and tumour-inducing plasmids share many similarities, including nearly identical organization of the vir operons (Moriguchi *et al.*, 2001). One exception is the absence of virE1 and virE2 from the Ri plasmid (and the genome) in some strains of *A. rhizogenes* (Moriguchi *et al.*, 2001; Hodges *et al.*, 2004). The single-stranded DNA (ssDNA)-binding protein VirE2 and its secretory chaperone VirE1 are critical for pathogenesis by
A. tumefaciens (Deng et al., 1999; Sundberg and Ream, 1999; Zhou and Christie, 1999). VirE2 is required only in plant cells; transgenic plants that produce VirE2 are fully susceptible to A. tumefaciens virE2 mutants (Citovsky et al., 1992). Inside plant cells, VirE2 protects T-strands from nuclease attack (Yusibov et al., 1994; Rossi et al., 1996) and helps promote their nuclear import (Zupan et al., 1996; Rossi et al., 1996; Gelvin, 1998). The genome of A. rhizogenes 1724 lacks virE1 and virE2 but still transfers T-strands efficiently due to the GALLS gene on the Ri plasmid (Hodges et al., 2004). The GALLS gene can complement an A. tumefaciens virE2 mutant, and the GALLS gene is essential for virulence in A. rhizogenes strains that lack virE1 and virE2 (Hodges et al., 2004). Full-length GALLS (GALLS-FL) (Hodges et al., 2006) and VirE2 (Vergunst et al., 2000; 2003; Simone et al., 2001) contain C-terminal signals for translocation into plant cells mediated by the VirB/D4 type IV secretion system (Fig. 1). VirE2 contains two NLSs (Citovsky et al., 1992; Zupan et al., 1996), whereas GALLS-FL contains a single bipartite NLS (Fig. 1) (Hodges et al., 2004), which is important for its ability to substitute for VirE2 (Hodges et al., 2006). This indicates that GALLS-FL performs a critical function inside the nucleus or at the nuclear membrane, as does VirE2.

Although GALLS-FL protein can substitute for VirE2 function, these proteins lack obvious similarities in their amino acid sequences. The closest known relatives of GALLS-FL are helicases and proteins involved in conjugative transfer of plasmids. The amino terminus of GALLS-FL resembles plasmid-encoded TraA (strand transferase) from A. tumefaciens and Sinorhizobium.
melloti (Farrand et al., 1996). This portion of GALLS-FL contains ATP-binding motifs (Walker boxes A and B) and a third motif found in members of a helicase/repliscope superfamily (Fig. 1) (Hodgman, 1988; Farrand et al., 1996), but VirE2 lacks these motifs. Changes in each motif abolish the ability of GALLS-FL to substitute for VirE2 (Hodges et al., 2006). The quantities of VirE2 and GALLS-FL produced by bacterial cells differ considerably. VirE2 is the most abundant virulence protein (Engstrom et al., 1987; Citovsky et al., 1988); coating a T-strand completely requires one molecule of VirE2 for every 20 bases of ssDNA (Frenkiel-Krispin et al., 2007). In contrast, GALLS-FL is present at very low levels in bacterial cells (Hodges et al., 2009). These obvious differences in quantities and biochemical activities strongly suggest that VirE2 and GALLS-FL promote nuclear import of T-strands via different mechanisms.

Biochemical properties of VirE2

VirE2 binds cooperatively to ssDNA

Agrobacterium tumefaciens transports VirE2, an abundant cooperative single-stranded DNA-binding (SSB) protein, into plant cells via the VirB/VirD4 type IV secretion system (Stachel and Zambrsksy, 1986; Yusibov et al., 1994; Ward and Zambrsksy, 2001). VirE2 export requires the secretory chaperone VirE1 (Sundberg et al., 1996; Deng et al., 1999; Zhou and Christie, 1999), a 65-residue acidic protein encoded by the first gene of the virE operon (Winans et al., 1987). VirE1 interacts with both C- and N-terminal domains of VirE2, preventing VirE2 from forming filamentous aggregates with itself (Sundberg et al., 1996; Deng et al., 1999; Zhou and Christie, 1999; Frenkiel-Krispin et al., 2007; Dym et al., 2008). VirE2 filaments retain the ability to bind ssDNA (Frenkiel-Krispin et al., 2007). The C-terminus of VirE2 is required for its export to plant cells via the VirB/VirD4 secretion system (Vergunst et al., 2000; Simone et al., 2001; Schrammeijer et al., 2003). VirE2 is critical for transformation of most host species, although specific tissues of some plant species (e.g. stems of Kalanchoe tubiflora; our unpublished data) eventually form small tumours after inoculation with a virE2 mutant (Garfinkel and Nester, 1980; Stachel and Nester, 1986). The VirE1 chaperone protein competes with ssDNA for binding to VirE2 (Frenkiel-Krispin et al., 2007; Dym et al., 2008) so that VirE2 binds T-strands only after export to plant cells (Ream, 1998; Cascales and Christie, 2004).

VirE2 functions in plant cells

VirE2 can bind T-strands from another bacterial cell

Mixed infection experiments suggest that A. tumefaciens lacking T-DNA may transport VirE2 directly into plant cells. Tumours form readily when a single plant wound is inoculated with two non-pathogenic strains of A. tumefaciens: one lacking T-DNA and the second mutant in virE2 (Otten et al., 1984; Christie et al., 1988; Sundberg et al., 1996). Both VirE2 and T-strand donors must contain wild-type virB and virD4 genes and chromosomal loci (chvA, chvB and exoC) necessary for binding to plant cells (Christie et al., 1988). Because both donors must be able to bind plant cells, VirE2 and T-strands are probably exported directly, and independently, into plant cells (Ream, 1998).

Transfer of other macromolecules through the VirB/VirD4 secretion system can interfere with VirE2 export. Mobilization of an IncQ plasmid (RSF1010) from A. tumefaciens via the VirB/VirD4 secretion system abolishes tumorigenesis by preventing secretion of VirE2 into plant cells (Binns et al., 1995; Stahl et al., 1998). In contrast, RSF1010 reduces but does not eliminate transfer of the T-complex (Stahl et al., 1998). The oncogenic suppressor protein Osa blocks export of VirE2 (but not the T-complex) into plant cells (Lee et al., 1999; Schrammeijer et al., 2003). Transfer of the T-complex can occur even though VirE2 export is blocked, confirming observations that VirE2 does not bind T-strands in bacterial cells (Cascales and Christie, 2004).

VirE2 is required only in plant cells

Direct evidence proves that the only important interaction between VirE2 and the T-strand-VirD2 complex (T-complex) occurs inside plant cells. Transgenic plants that produce VirE2 are susceptible to transformation by virE-mutant A. tumefaciens, proving that VirE2 is required only in plant cells (Citovsky et al., 1992; Gelvin, 1998; Bhattacharjee et al., 2008).

VirE2 interacts with host proteins involved in nuclear targeting

VirE2 interacts with several plant proteins, including VIP1 and VIP2 (Gelvin, 2000; Tzfira et al., 2001; 2002; Ward and Zambrsksy, 2001; Tzfira and Citovsky, 2002; Ward et al., 2002) and members of the importin α family (Bhattacharjee et al., 2008). VIP1 and importin α mediate nuclear import of VirE2, which contains two NLSs (Citovsky et al., 1992; Tzfira et al., 2001). Although several members of the importin α family can interact with VirE2, importin α 4 is the only importin required for efficient transformation of Arabidopsis thaliana roots (Bhattacharjee et al., 2008). The NLSs in VirE2 differ from the NLS in VirD2, which binds AtKAPα (importin α 1) (Ballas and Citovsky, 1997; Tzfira et al., 2001; Ward and Zambrsksy, 2001; Ziemienowicz et al., 2001; Tzfira and Citovsky, 2002) and other members of the importin α family (Bhattacharjee et al., 2008). The VirD2 NLS functions in animal
cells, but the VirE2 NLSs do not (Guralnick et al., 1996; Relic et al., 1998; Ziemienowicz et al., 1999; Rhee et al., 2000; Tzfira et al., 2000). VIP2 is similar to Rga, which mediates interactions between chromatin proteins and transcription complexes (in Drosophila melanogaster) (Frolov et al., 1998; Tzfira et al., 2000; Ward and Zambryski, 2001). VIP2 may escort the VirE2-bound T-strands to chromatin during T-DNA integration (Tzfira et al., 2000; Ward and Zambryski, 2001).

**Roles of VirE2 in plant cells**

**VirE2 SSB protein protects T-strands**

Inside plant cells, VirE2 protects T-strands from nuclease attack. A virE2 mutation drastically reduces the amount of T-strands recovered from the cytoplasm of infected plant cells, even though T-strand levels in bacterial cells remain normal (Stachel et al., 1987; Veluthambi et al., 1988; Yusibov et al., 1994; Gelvin, 1998). Although virE2 null mutations severely reduce tumorigenesis, rare transformation of plant cells occurs (our unpublished data) (Garfinkel and Nester, 1980; Stachel and Nester, 1986). In the absence of VirE2, integrated T-DNAs are often truncated at their left ends, confirming the importance of VirE2 in the absence of VirE2, integrated T-DNAs are often truncated at their left ends, confirming the importance of VirE2 for protection of T-strands from nuclease attack (Yusibov et al., 1994; Rossi et al., 1996). Thus, T-strands are more susceptible to degradation in the absence of VirE2.

**VirE2 forms pores in artificial membranes**

VirE2 may also form a channel through the plant plasma (or nuclear) membrane. In vitro, VirE2 can insert into a lipid bilayer and form a voltage-gated channel that permits ssDNA to pass through the artificial membrane (Dumas et al., 2001; Duckely and Hohn, 2003). We do not know whether VirE2 forms transmembrane pores in plant cells.

**Localization of VirE2 expressed in plant cells**

VirE2 expressed at high levels directly in plant cells in the absence of VirD2 and T-strands may behave differently than VirE2 translocated from A. tumefaciens into plant cells through normal channels and in proper amounts. VirE2 produced in plant cells is present in both the nucleus and cytoplasm. In several studies, reporter-tagged VirE2 accumulated predominantly at the nucleus in plant cells (Citovsky et al., 1992; 1994; 2004; Tzfira et al., 2001; Ziemienowicz et al., 2001; Li et al., 2005), although some remained in the cytoplasm (Citovsky et al., 1992). Another study detected VirE2 at the nucleus, in cytoplasmic strands, and at the cell periphery (Grange et al., 2008). A recent study showed cytoplasmic accumulation of tagged VirE2, although bimolecular fluorescence complementation showed that VirE2 interacts with importin α 4 primarily at the nucleus (Bhattacharjee et al., 2008). VirE2 expressed in transgenic plants can restore virulence to A. tumefaciens virE2 mutants (Citovsky et al., 1992; Bhattacharjee et al., 2008), suggesting that VirE2 may function from its nuclear location in these transgenic cells.

**VirE2 targets T-strands to the nucleus**

VirE2 promotes localization of T-strands to the nucleus. The central region of VirE2 contains both NLSs (Citovsky et al., 1992) (Fig. 1). The NLSs overlap regions that are important for binding ssDNA and for cooperative interaction between VirE2 molecules (Citovsky et al., 1992; Dombek and Ream, 1997; Sundberg and Ream, 1999) (Fig. 1). The NLSs can interact with importin α 4 (Bhattacharjee et al., 2008) and promote nuclear targeting when VirE2 binds to ssDNA (Zupan et al., 1996), despite the involvement of these regions in protein–DNA and protein–protein interactions (Dym et al., 2008). Fluorescently-labelled ssDNA coated with VirE2 (but lacking VirD2) accumulates at the nucleus upon microinjection of the complex into plant cells (Zupan et al., 1996). In this study, the intracellular location of VirE2-bound ssDNA was followed by epifluorescence microscopy, which cannot determine whether the VirE2–ssDNA complex is imported into the nucleus or whether it remains bound to the nuclear membrane. Thus, VirE2 can direct ssDNA to the nucleus.

**VirD2 and VirE2 promote nuclear import of T-strands**

The NLS near the C-terminus of VirD2 (Fig. 1) plays an important role in nuclear import of T-strands (Rossi et al., 1993). A deletion that removes this NLS and a flanking region involved in binding importin α 4 (Bhattacharjee et al., 2008) reduces virulence to ~1% of wild type (Shurvinton et al., 1992). A viral NLS (with a very different amino acid sequence) can substitute for the VirD2 NLS, indicating that nuclear targeting is important for VirD2 function in vivo (Shurvinton et al., 1992).

VirD2 initiates import of ssDNA into nuclei of permeabilized tobacco protoplasts; rhodamine-labelled 25-nucleotide ssDNA molecules with VirD2 covalently attached to the 5′ end are imported into the nucleus in an NLS-dependent manner, as determined by confocal microscopy, whereas free ssDNA remains outside the nucleus (Ziemienowicz et al., 2001). Longer VirD2-bound ssDNA molecules (250 or 1000 nucleotides) do not enter the nucleus in the absence of VirE2, but addition of VirE2 promotes nuclear import of these VirD2–ssDNA complexes (Ziemienowicz et al., 2001). Thus, VirD2 and VirE2 collaborate to translocate ssDNA into the nucleus.
Single-stranded DNA interferes with nuclear localization of VirE2

In the permeabilized tobacco protoplast system, free VirE2 (in the absence of ssDNA) accumulates in the nucleus, but VirE2–ssDNA complexes (in the absence of VirD2) remain outside the nucleus (Ziemienowicz et al., 2001). Apparently, binding to ssDNA interferes with nuclear localization of VirE2. These authors suggested that the NLSs of VirE2 may be unavailable to the nuclear import machinery when VirE2 is bound to ssDNA (Ziemienowicz et al., 2001). Alternatively, complexes of VirE2 bound to long ssDNA molecules may be too large to pass through the nuclear pore, even if the NLSs remain accessible when VirE2 binds ssDNA, as suggested by two recent studies. VirE2 can bind importin α 4 and ssDNA simultaneously (Bhattacharjee et al., 2008), and the X-ray crystal structure of VirE2 predicts that the NLSs of VirE2 face the exterior of the solenoid-shaped complex formed between ssDNA and VirE2 (Dym et al., 2008). The observation that VirE2–ssDNA complexes are unable to enter the nucleus suggests that free VirE2 located in the nucleus prior to DNA binding (rather than cytoplasmic VirE2) may mediate nuclear import of T-strands. This is consistent with studies that show VirE2 accumulates predominantly at the nucleus of plant cells (Citovskev et al., 1992; 1994; 2004; Tzfrira et al., 2001; Ziemienowicz et al., 2001; Li et al., 2005; Grange et al., 2008).

RecA protein can mediate nuclear import of VirD2–ssDNA complexes in vitro

The RecA recombinase (of Escherichia coli) can substitute for VirE2 in nuclear import of VirD2–ssDNA complexes in permeabilized tobacco cells (Ziemienowicz et al., 2001). RecA is a cooperative SSB protein that localizes to the nucleus and cytoplasm of the permeabilized plant cells. RecA polymerizes on ssDNA in a 5′ to 3′ direction in an ATP-dependent process (Anderson and Kowalczykowski, 1997; Churchhill et al., 1999). VirD2 bound to ssDNA is able to transport ~25 nucleotides at the 5′ end into the nucleus (Ziemienowicz et al., 2001), and subsequent ATP-dependent cooperative binding by RecA protein may provide sufficient energy to pull the ssDNA completely into the nucleus.

Cooperative binding of VirE2 to ssDNA may pull VirD2-bound T-strands into the nucleus

Cooperative binding by VirE2 may also provide the energy required to transport ssDNA into the nucleus. VirE2, (presumably already inside the nucleus) may bind to the 5′ end of ssDNA that has been partially transported into the nucleus by VirD2. Although VirE2 lacks ATPase activity, cooperative binding of VirE2 to the 5′ end of ssDNA–VirD2 complexes may generate sufficient force to pull the remaining ssDNA completely inside the nucleus. Indeed, as VirE2 binds ssDNA cooperatively (Gietl et al., 1987; Christie et al., 1988; Citovsky et al., 1988; 1989; Das, 1988; Sen et al., 1989), it can pull the ssDNA without requiring external energy such as ATP hydrolysis (Grange et al., 2008).

A new model for nuclear import of T-strands

The following hypothesis summarizes the current evidence regarding the roles of VirD2 and VirE2 in nuclear import of T-strands, and the model (Fig. 2) makes testable predictions for future research. Nuclear import of T-strands may begin when VirD2 enters the nucleus along with ~25 nucleotides of covalently attached ssDNA, which is sufficient to accommodate one molecule of VirE2 (Dym et al., 2008). Then VirD2 may recruit a molecule of free VirE2 (already localized inside the nucleus) to the 5′ end of the T-strand. Protein interactions direct other DNA-binding proteins to sites where they are needed. For example, purified RecA recombinase binds ssDNA in vitro (Radding, 1991; Kowalczykowski et al., 1994), but the RecOR complex or the RecBCD enzyme help load RecA onto ssDNA in vivo (Anderson and Kowalczykowski, 1997; Webb et al., 1997; Churchhill et al., 1999; Kantake et al., 2002; Morimatsu and Kowalczykowski, 2003). Similarly, this model predicts that VirD2 will interact with VirE2 inside plant nuclei and stimulate cooperative binding of VirE2 to the 5′ end of the T-strand in a 5′ to 3′ direction, thereby pulling T-strands completely inside the nucleus.

The A. rhizogenes GALLS gene substitutes for virE2

Despite the importance of VirE2 for nuclear import of T-strands during A. tumefaciens-mediated gene transfer to plants, VirE2 is completely dispensable for GALLS-dependent A. rhizogenes-mediated gene transfer. Thus, GALLS-encoded proteins must provide an alternate means to promote nuclear import of T-strands. The ability of the abundant VirE2 protein to protect T-strands from nuclease attack by fully coating T-strands is also dispensable during GALLS-mediated gene transfer; small quantities of GALLS-FL protein are sufficient to promote efficient gene transfer.

The GALLS gene encodes two proteins

The GALLS gene encodes a full-length protein of 1769 amino acids (GALLS-FL) and a C-terminal domain (GALLS-CT) of 962 amino acids (Fig. 1) (Hodges et al., 2009). Translation of GALLS-CT initiates at an internal in-frame start codon (Met808), which is required for
production of GALLS-CT (Hodges et al., 2009). On some hosts, both GALLS proteins are required to substitute for VirE2, but on others GALLS-FL is sufficient (Hodges et al., 2009). Most of GALLS-CT consists of nearly identical 266-residue sequences repeated three times (Fig. 1). Mutant GALLS-FL and GALLS-CT proteins that contain a single copy of the repeat sequence are unable to substitute for VirE2 effectively, although the proteins remain stable (Hodges et al., 2004).

**GALLS belongs to a family of helicases and primases involved in conjugation**

Other genes that encode translational restart proteins include mobA of RSF1010 (Scholz et al., 1989), the primases of plasmids Coll (sog) (Boulnois et al., 1982) and R16 (pri) (Dairymple and Williams, 1984), phage T7 gene 4 primase/helicase (Dunn and Studier, 1981), and cisA nickase/helicase of φX174 (Linney and Hayashi, 1974). RSF1010 mobA produces full-length MobA (relaxase/primase) and RepB (primase), which is identical to the C-terminal portion of MobA; this sequence also encodes MobB, another relaxase subunit, from a different internal reading frame (Scholz et al., 1989). These genes share intriguing similarities with GALLS, including their involvement in plasmid conjugation (mobA, sog and pri) and the enzymatic activities they encode (cisA and gene 4 helicases). The translational restart proteins encoded by sog, pri and cisA are more abundant than the corresponding full-length proteins (Linney and Hayashi, 1974; Boulnois et al., 1982; Dairymple and Williams, 1984), as is the case for the GALLS-encoded proteins (Hodges et al., 2009). Although the functions of these restart proteins are unknown, their abundance suggests a structural role rather than an enzymatic activity (Boulnois et al., 1982).

The low abundance of GALLS-FL may result from the high incidence of rarely used leucine codons upstream of the translation start codon for the more abundant GALLS-CT protein (Hodges et al., 2009). Lower pools for tRNAs that recognize these rare codons may limit translation of GALLS-FL. Both GALLS proteins are expressed from a single promoter, and mRNA levels are similar at both translation start codons, which are preceded by identical ribosome binding sites (AGGAG) and a favoured A at −3 (Hodges et al., 2009). Reduced translation of GALLS-FL due to codon bias or instability of GALLS-FL protein may account for its low abundance.

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**Fig. 2.** Model for VirE2-mediated nuclear import of T-strands. The green circle represents a plant cell plasma membrane, and the blue circle represents the nuclear membrane. Inside the nucleus, the solid green circle labelled ‘D2’ depicts a molecule of VirD2 covalently attached to the 5’ end of the T-strand (red line). The yellow ovals labelled ‘VirE2’ represent VirE2 protein.

A. VirD2 enters the nucleus along with < 250 nucleotides of attached T-strand. VirE2 monomers enter the nucleus separately, and VirD2 recruits a VirE2 monomer to the 5’ end of the T-strand.

B. The VirE2 monomer bound to VirD2 and the T-strand recruits a second molecule of VirE2, pulling additional nucleotides of the T-strand into the nucleus.

C. Cooperative binding of additional VirE2 molecules to the T-strand in the 5’ to 3’ direction pulls more ssDNA into the nucleus.

D. Nuclear import is complete and the T-strand is fully coated with VirE2.
The C-terminal region of GALLS contains a protein interaction domain

GALLS-CT contains a protein interaction domain that promotes self-interaction and binding to GALLS-FL and VirD2. Because GALLS-CT is much more abundant than GALLS-FL in bacterial cells, it may bind GALLS-FL and prevent premature interaction of GALLS-FL with VirD2 prior to export from bacterial cells. Similarly, GALLS-CT may prevent self-aggregation of GALLS-FL in bacterial cells. Both GALLS proteins contain identical type IV secretion signals and probably are translocated into plant cells with equal efficiency, thereby maintaining an excess of GALLS-CT relative to GALLS-FL in the cytoplasm of the plant cell. However, GALLS-CT is excluded from the nucleus unless it interacts with either VirD2 or GALLS-FL. Therefore, inside the nucleus, the level of GALLS-CT may be lower than in the cytoplasm. Once inside the nucleus, GALLS-CT may be displaced allowing GALLS-FL and VirD2 to assemble into a complex at the 5′ end of the T-strand, or all three proteins may form a multi-subunit complex. GALLS-CT also may modulate the predicted helicase activity of GALLS-FL, or it may anchor the DNA–protein complex to host proteins in the nucleus. Alternatively, high levels of GALLS-CT may saturate proteases that would otherwise degrade GALLS-FL.

Localization of GALLS proteins in plant cells

GALLS-FL expressed in tobacco protoplasts localizes inside the nucleus in an NLS-dependent manner, whereas GALLS-CT expressed separately remains in the cytoplasm because GALLS-CT lacks the NLS (Hodges et al., 2009). However, when the two GALLS proteins are expressed together, they interact with each other and accumulate inside the nucleus (Hodges et al., 2009). As expected, GALLS-FL interacts with importin α 4 and localizes inside the nucleus (Hodges et al., 2009).

GALLS-FL interacts with the VirD2 pilot protein

VirD2 interacts with GALLS-FL and localizes inside the nucleus (Hodges et al., 2009), suggesting that VirD2 may recruit GALLS-FL to the leading (5′) end of the T-strand inside the nucleus. GALLS-FL protein compensates for the absence of VirE2, apparently without duplicating its activities.

A model for GALLS-mediated nuclear import of T-strands

VirE2 is an abundant SSB protein required in stoichiometric amounts to coat and protect T-strands (Yusibov et al., 1994; Rossi et al., 1996) and perhaps promote their nuclear import (Yusibov et al., 1994; Rossi et al., 1996; Zupan et al., 1996; Gelvin, 1998). In contrast, GALLS-FL is likely a low-abundance enzyme that may mobilize T-strands into the nucleus using its predicted ATP-dependent strand transferase/helicase activity (Hodges et al., 2006). GALLS-FL requires its NLS to function (Hodges et al., 2006) and localizes inside the nucleus of host cells (Hodges et al., 2009), suggesting that it provides an alternative means to transport T-strands into the nucleus. GALLS-FL and VirD2 can interact when they are co-expressed in plant cells, and this complex localizes inside the nucleus (Hodges et al., 2009). Thus, during T-strand transfer, GALLS-FL may be anchored to VirD2 at the leading (5′) end of the T-strand. GALLS-FL has a predicted helicase domain and may translocate along T-strands in a 5′ to 3′ direction, disrupting secondary structures that may form in T-strands in the absence of VirE2 SSB. If the helicase remains in a fixed position, translocation along DNA would cause the DNA to move. Thus, GALLS-FL may pull T-strands into the nucleus (Fig. 3), obviating the need for VirE2 to mediate nuclear import of T-strands. Extensive degradation of T-strands observed in plant cells infected with virE2-mutant A. tumefaciens (Yusibov et al., 1994; Rossi et al., 1996) may occur because progress of T-strands into the nucleus is stalled in plant cells lacking both VirE2 and GALLS-FL. Efficient nuclear import of T-strands in the presence of either VirE2 or GALLS-FL may minimize opportunities for nuclease attack in the cytoplasm.

Evolution of a novel effector protein-secretion system combination

The GALLS gene illustrates the evolution of a novel type IV secretion system–effector protein combination that may confer the ability to mobilize bacterial DNA into the nucleus of eukaryotic cells. To compensate for loss of the T-DNA to plant cells. The GALLS gene adjoins conjugation (tra) genes ~60 kb away from the nearest vir gene. Thus, a promiscuous gene transfer system capable of delivering DNA to eukaryotic cells apparently evolved from a type IV secretion system and a bacterial conjugation system.

Implications for plant biotechnology

Advantages of Agrobacterium-mediated plant transformation

Plant molecular biologists created ‘disarmed’ strains of A. tumefaciens that lack oncogenes but retain virulence (vir) genes needed to transfer genes into plants (Gelvin,
2003). This technology is widely used to create transgenic plants for research and biotechnology. Transgenes delivered using *A. tumefaciens* vectors have lower copy numbers and undergo fewer rearrangements than those in plants transformed by other technologies. Bacterial virulence proteins are transported to plants and help target the T-DNA to the nucleus and maintain its integrity during integration into the genome, making *A. tumefaciens* the preferred method to deliver genes to plants.

VirE2- and GALLS-mediated transformation systems may require different host factors

Although VirE2 and GALLS both interact with host importin α 4 (Bhattacharjee et al., 2008; Hodges et al., 2009), these proteins may also interact with different host proteins. For example, VirE2 interacts with VIP1 and VIP2 (Gelvin, 2000; Tzfira et al., 2001; 2002; Ward and Zambryski, 2001; Tzfira and Citovsky, 2002; Ward et al., 2002), but we do not know whether these VIP proteins interact with the GALLS proteins. Because VirE2 and GALLS-FL do not share amino acid sequence similarity and have different functional domains and modes of action, it seems likely that VirE2 and GALLS-FL will interact with different host proteins, which almost certainly influence transformation efficiency. Thus, one transformation system may perform more efficiently than the other on plant species that are recalcitrant to *Agrobacterium*-mediated transformation. Host factors that limit transformation by one system may not be required by the other. Thus, GALLS-mediated transformation may alter the efficiency of transformation. Also, GALLS-mediated transformation may alter the average transgene copy number or reduce the frequency of rearranged transgenes relative to VirE2-promoted events.

**GALLS-mediated transformation may facilitate transgene integration by homologous recombination**

GALLS-mediated gene transfer may allow gene replacement in plants by homologous recombination. Gene replacement by homologous recombination is the ‘holy grail’ of plant science and biotechnology. *Agrobacterium tumefaciens*-mediated transfer of DNA into plant cells precludes homologous recombination between the incoming DNA and the host genome, except for very rare events (Kempin et al., 1997; Shaked et al., 2005). VirE2 coats T-strands and likely prevents T-strands from interacting with recombination enzymes. This may explain the inability to integrate VirE2-bound T-DNA by homologous...
recombination at a useful frequency. Instead, T-DNA is normally integrated by non-homologous end-joining (Offringa et al., 1990; Mayerhofer et al., 1991; van Attikum et al., 2001). Sometimes T-DNA can be targeted to specific sites in the genome, but the inability to move genes into chromosomes by homologous recombination is a serious limitation. GALLS-mediated transfer differs from VirE2-mediated events. GALLS-FL is not abundant enough to coat the entire length of T-strands, so the DNA may remain available for homologous recombination.

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