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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* (*ro*ot *l*oci) 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

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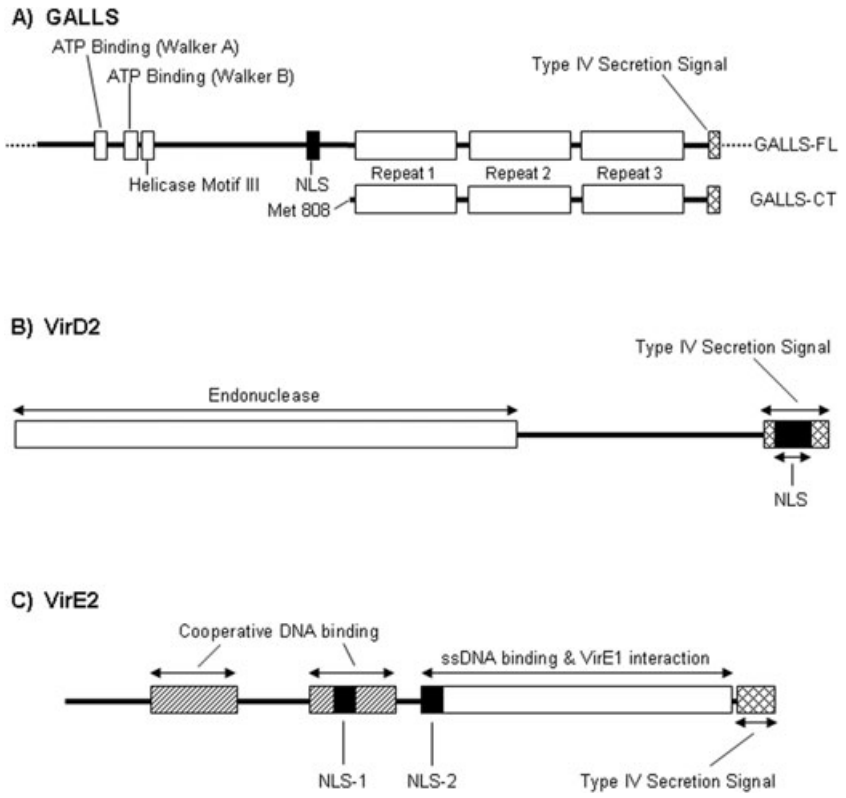

Fig. 1. Domains in the GALLS, VirD2 and VirE2 proteins.
A. GALLS-FL and GALLS-CT proteins. Boxes indicate the locations of the ATP binding sites (Walker A & B; [165]V**G**VA**GS**A**KT**S[174] & [235]**IVVIDE**MSM[243]), helicase motif III ([269]**KLI**C**VGD**DR**QL**P**PV**GP**G**DLL[288]), nuclear localization signal (NLS; [705]**KRKR**AAA**K**EEIDS**RKK**MA**RH**[724]), GALLS repeats [amino acids 828–1093 (repeat 1); 1117–1382 (repeat 2); 1406–1671 (repeat 3)], and type IV secretion signal ([1743]PKAANDVD**R**LTRDFDE**R**I**R**V**R**GDGRGL[1769]; consensus sequence: **R**xxxxxxx**R**x**R**x**R**xx). Bold type indicates basic amino acids in the NLS or amino acids that match the corresponding consensus sequences in the ATP binding sites, helicase motif III, and type IV secretion signal. The *GALLS* gene encodes a full-length protein (GALLS-FL; top bar) and a C-terminal domain (GALLS-CT; bottom bar), which results from translation initiated at an in-frame start codon (Met 808).
B. VirD2 functional domains. The N-terminal region contains the endonuclease domain (open box; amino acids 1–229). The NLS (black box; [396]**KR**P**R**D**RH**DGELGG**RKRA**R[413]) lies within the type IV secretion signal (hatched box; [395]PKRPRDRHDGELGGRKRA**R**GNR**R**DDGRGGT[424]). Arrows indicate the extent of each domain.
C. VirE2 functional domains. VirE2 contains two domains for cooperative ssDNA binding (forward hashed boxes) and two NLS sequences (black boxes; NLS-1: [205]**KLR**PED**R**YVQTE**K**YG**RR**[221] and NLS-2: [273]**KRR**YGGETI**KLKSK**[287]). NLS-1 lies within a cooperativity domain, and NLS-2 lies within a domain (amino acids 273–495) required for binding ssDNA and for interaction with VirE1, the secretory chaperone for VirE2. The type IV secretion signal occupies the C-terminus (hatched box; [504]FVRPEPASRPISDSRRIYES**R**P**R**SQSVNSF[533]).

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*

meliloti (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/replicase 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 Zambryski, 1986; Yusibov *et al.*, 1994; Ward and Zambryski, 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 tubiflorae*; 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 Zambryski, 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 Zambryski, 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 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 (Citovsky *et al.*, 1992; 1994; 2004; Tzfira *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; Churchill *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; Churchill *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

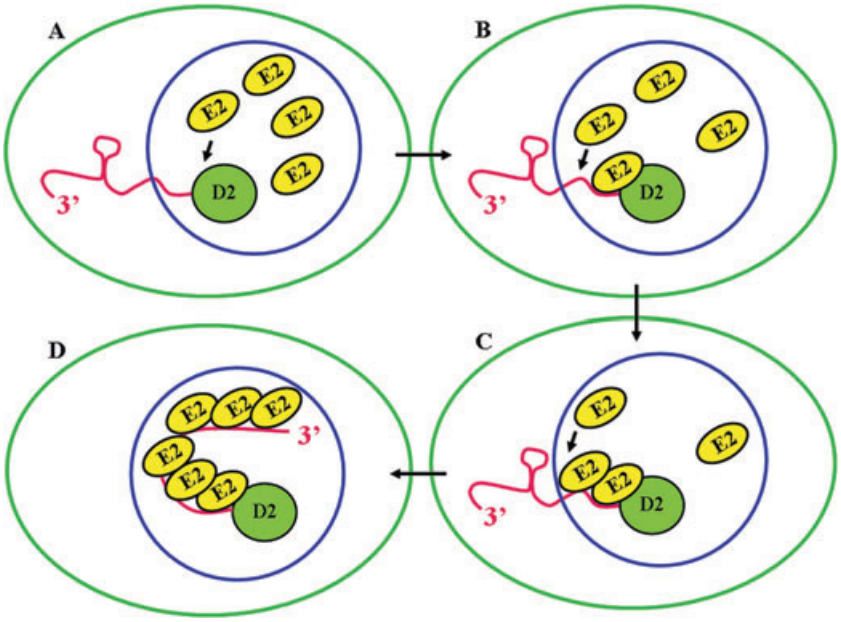

Fig. 2. Model for VirE2-mediated nuclear import of T-strands. The green circle represents a plant cell plasma membrane, and the blue circle represents the nuclear membrane. Inside the nucleus, the solid green circle labelled 'D2' depicts a molecule of VirD2 covalently attached to the 5′ end of the T-strand (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.

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 ColI (*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.

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 GALL-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 *virE1* and *virE2*, *A. rhizogenes* appropriated an unusual conjugation gene (*GALLS*) to restore its ability to deliver 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,

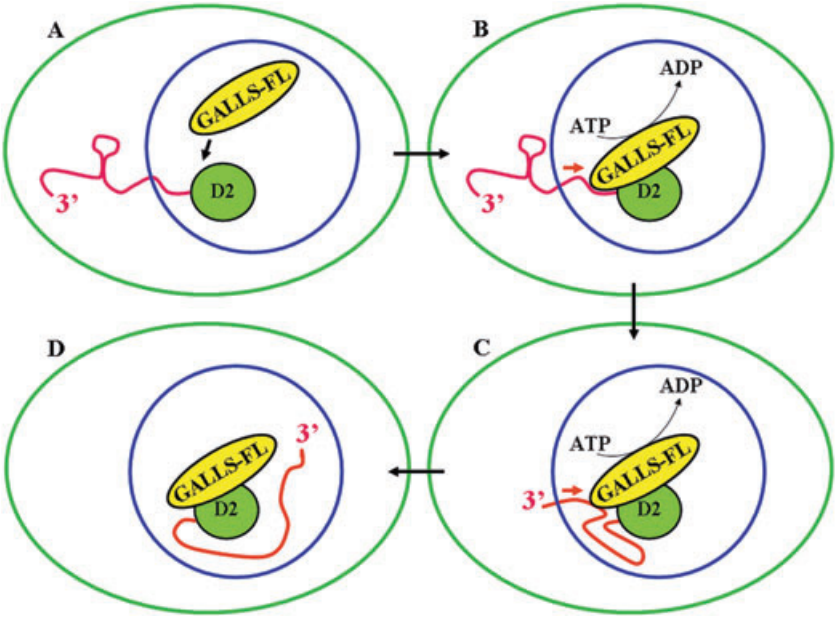

Fig. 3. Model for GALLS-mediated nuclear import of T-strands. The green circle represents the plant cell plasma membrane, and the blue circle represents the nuclear membrane. Inside the nucleus, the solid green circle labelled 'D2' depicts a molecule of VirD2 covalently attached to the 5′ end of the T-strand (red line). The yellow oval labelled 'GALLS-FL' represents GALLS-FL protein.
A. VirD2 enters the nucleus along with < 250 nucleotides of attached T-strand. GALLS-FL enters the nucleus separately, and VirD2 recruits GALLS-FL to the 5′ end of the T-strand.
B. ATP-dependent translocation of GALLS-FL protein in the 5′ to 3′ direction along the T-strand pulls additional nucleotides of T-strand into the nucleus.
C. GALLS-FL continues to pull the T-strand into the nucleus, using its predicted helicase activity to disrupt any base pairs that form between complementary sequences in the T-strand.
D. Nuclear import of the T-strand is complete.

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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