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Finding a way to the nucleus

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Agrobacterium species transfer single-strand DNA and virulence effector proteins to plants. To understand how *Agrobacterium* achieves interkingdom horizontal gene transfer, scientists have investigated how the interaction of bacterial effector proteins with host proteins directs T-DNA to the plant nucleus. VirE2, a single-strand DNA binding protein, likely plays a key role in T-DNA nuclear targeting. However, subcellular trafficking of VirE2 remains controversial, with reports of both cytoplasmic and nuclear localization. The recent discovery that phosphorylation of the VirE2 interacting protein VIP1 modulates both nuclear targeting and transformation may provide a solution to this conundrum. Novel experimental systems that allow tracking of VirE2 as it exits *Agrobacterium* and enters the plant cell will also aid in understanding virulence protein/T-DNA cytoplasmic trafficking.

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Introduction

Agrobacterium species, 'nature's genetic engineer', are well-known mediators of interkingdom horizontal gene transfer. These phytopathogens cause neoplastic growths on host plants (crown gall and hairy root disease), but are best known as agents for generating transgenic plants [1]. *Agrobacterium* transports single-strand transferred DNA (T-DNA, Box 1) to plants through a bacterial type IV protein secretion system (T4SS). Processing of T-DNA is initiated by VirD2, which nicks the tumor inducing (Ti) plasmid at T-DNA border repeat sequences. During this cleavage reaction, VirD2 covalently attaches to single-strand T-DNA (termed the 'T-strand') at its 5′ end. VirD2 is thought to pilot T-strands through the T4SS, into the plant cell, and to the nucleus. VirD2/T-strands are not the only molecules transferred to the plant via the T4SS. Other transferred *Agrobacterium* effector proteins include VirD5, VirE2, VirE3, and VirF (for reviews, see [2,3,4•]). Transfer of effector proteins to host cells through a T4SS is also important for animal and human pathogenesis by a number of bacteria, including *Helicobacter*, *Brucella*, *Bordetella*, *Bartonella*, and *Legionella* species [5].

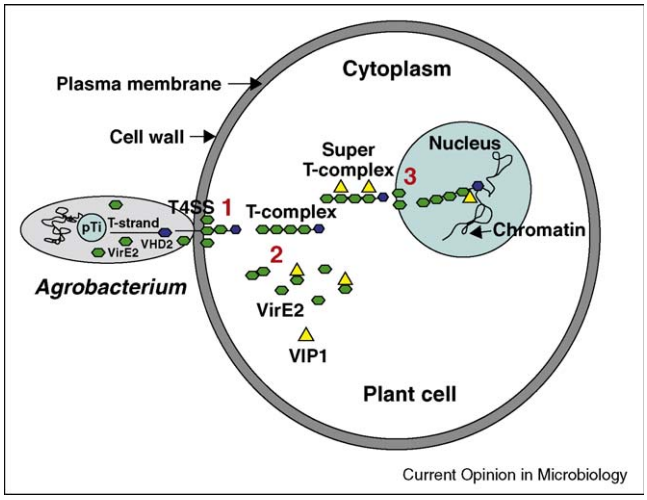
In addition to VirD2, of special importance for plant genetic transformation is VirE2, a single-strand DNA binding protein. VirE2 binds cooperatively to single-strand DNA *in vitro* [6–8] and is hypothesized to bind to T-strands in the plant, thus protecting the DNA from nucleolytic destruction [9,10]. The complex of T-strands covalently linked to VirD2 and coated by VirE2 molecules is termed the 'T-complex' [11]. Although there are strong genetic and *in vitro* binding data indicating the existence of the T-complex, such a complex has not been demonstrated in plants.

Interaction of VirD2/T-strands and VirE2 with other secreted *Agrobacterium* virulence effector proteins and plant proteins likely generates 'super-T-complexes' that are responsible for subcellular trafficking of T-strands from the plant cell wall and membrane through the cytoplasm, into the nucleus, and to chromatin, thus facilitating T-DNA integration into the plant genome. VirD2 interacts with several plant cyclophilins [12,13], all tested importin α isoforms [12,14,15••], the kinase CAK2Ms [12], a TATA box binding protein [12], and the protein phosphatase PP2C [16]. Interaction with importin α and phosphorylation by PP2C are important for nuclear targeting of VirD2, whereas interaction with CAK2Ms and the TATA box binding protein may be important for targeting of VirD2/T-strands to chromatin [12]. VirE2 also interacts with several importin α isoforms [15••], as well as with two *V*irE2 *i*nteracting *p*roteins VIP1 and VIP2 [17]. Interaction of VIP1 with histones and nucleosomes may mediate targeting of T-strands to plant chromatin [18•,19]. VIP2 may facilitate T-DNA integration into the genome: *vip2* mutant *Arabidopsis* plants support transient but not stable *Agrobacterium*-mediated transformation [20]. Because VirE2 molecules likely 'coat' T-strands and also interact with plant proteins that help effect nuclear targeting, the subcellular localization of VirE2 is crucial to understanding cytoplasmic trafficking of T-strands. Figure 1 presents several models describing the role of VirE2 in targeting T-strands to the nucleus. These models, and the data supporting them, are discussed below.

Nuclear targeting of important *Agrobacterium* virulence effector proteins

Both VirD2 and VirE2 contain nuclear localization signal (NLS) sequences that interact with importin α proteins.

Figure 1



Models describing the possible roles of VirE2 in targeting T-strands to the plant nucleus. T-strands are processed from the Ti-plasmid by the action of the VirD2 endonuclease, which covalently links to the 5′ end of T-strands and pilots them through the *Agrobacterium* T4SS and into the plant cell. VirE2 protein separately enters the plant cell through the T4SS. In Model 1, VirE2 initially remains in the plant plasma membrane and 'picks up' T-strands as they enter the plant cell [47]. In Model 2, VirE2 enters the plant cytoplasm and interacts with T-strands and VIP1 protein, forming the T-complex. Super-T-complexes can form when T-complex proteins further interact with other secreted *Agrobacterium* virulence effector proteins, and/or with plant-encoded proteins such as VIP1 [17] and importin α [15••]. Nuclear targeting of super-T-complexes may depend upon the phosphorylation status of VIP1 [33••]. In Model 3, VirE2 enters the nuclear membrane, interacts with VirD2 molecules on the T-strands, and helps 'pull' T-strands into the nucleus [46••]. VIP1 may subsequently target super-T-complexes to chromatin to facilitate T-DNA integration into the plant genome.

Box 1 Definitions

Ti-plasmid	Tumor inducing plasmid found in virulent *A. tumefaciens* strains
Ri-plasmid	Root-inducing or rhizogenic plasmid found in virulent *A. rhizogenes* strains
T-DNA	Transferred DNA; DNA transferred from *Agrobacterium* to plants
T-strand	Single-strand form of T-DNA transferred to plants
Vir protein	Virulence protein; some are effector proteins transferred to plants
T4SS	Type IV secretion system; used to transfer T-strands and Vir effector proteins to plants
T-complex	Complex formed by VirD2 attached to the 5′ end of T-strands, and VirE2 coating the T-strands
Super-T-complex	T-complex in association with other Vir effector proteins and plant proteins
VIP1/VIP2	VirE2 interacting proteins 1 and 2
NLS	Nuclear localization signal sequence
GUS	β-Glucuronidase
HA	Hemagglutinin
YFP/CFP	Yellow/Cyan fluorescence protein
VirE2i–cCFP	VirE2 protein internally tagged with the C-terminal fragment of CFP
GALLS-FL	Full-length GALLS protein
GALLS-CT	C-terminal region of GALLS protein generated by translational restart within the *GALLS* gene

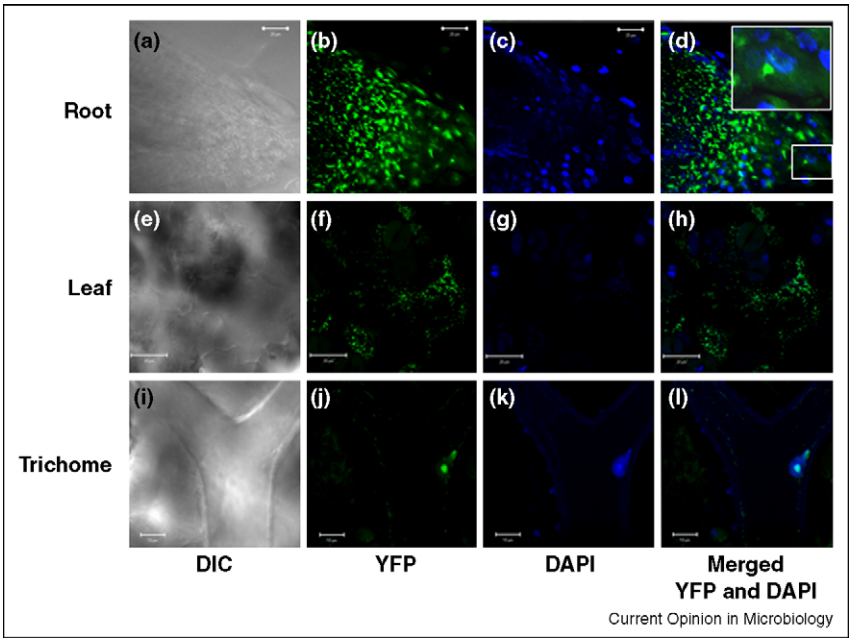
The bipartite VirD2 NLS sequence near the carboxy-terminus can direct affixed reporter proteins to the nucleus in plant, animal, and yeast cells [21–26]. Thus, it is likely that VirD2 helps direct covalently linked T-strands to the plant nucleus. The subcellular localization of VirE2 remains, however, controversial. Several reports demonstrated nuclear localization of VirE2. Conversely, several other laboratories have demonstrated cytoplasmic localization of VirE2.

All published VirE2 subcellular localization studies to date have involved VirE2 (or VirE2–single-strand DNA complexes) 'artificially' introduced into plant cells. Thus, when VirE2 bound to fluorescently labeled single-strand DNA was microinjected into *Tradescantia* stamen hair cells, fluorescence localized predominantly in the nucleus [27]. In another study, however, fluorescently labeled single-strand DNA bound to VirE2 was introduced into permeabilized tobacco cells and remained in the cytoplasm, despite the fact that in these experiments VirE2 protein alone localized in the nucleus [28]. Only when long fluorescently labeled single-strand DNA molecules were both linked to VirD2 and coated with VirE2 could the DNA enter the nucleus. Thus, the role of VirE2 in targeting T-strands to the nucleus remains unresolved.

Other studies in which tagged VirE2 protein was introduced into plant cells yielded similarly conflicting results: when an appropriate expression cassette was introduced by microprojectile bombardment, a GUS–VirE2 fusion protein localized predominantly in the nucleus in maize leaves and immature roots, and in tobacco immature roots, but exclusively in the cytoplasm in mature maize and tobacco roots [25]. A similar construction, when bombarded into tobacco leaf mesophyll cells and onion root epidermal cells, resulted in exclusively nuclear localization of GUS activity [17,29]. These results suggest that the subcellular localization of VirE2 may depend upon the developmental status of the particular plant tissue under investigation. Indeed, recent results from this author's laboratory indicate that VirE2 localizes to different subcellular compartments in different tissues. Figure 2 shows that YFP fluorescence in transgenic *Arabidopsis* plants expressing a VirE2–YFP fusion protein (that retains VirE2 functional activity) localizes exclusively in the cytoplasm of root, leaf epidermal, and leaf guard cells, but predominantly in the nucleus of leaf trichomes.

Although predominantly nuclear localization of VirE2 was observed in earlier studies, other groups have more recently demonstrated cytoplasmic localization of VirE2. VirE2–HA [30•] localized exclusively in the cytoplasm of bombarded tobacco BY-2 cells, and VirE2–YFP similarly

Figure 2



Localization of VirE2–YFP in transgenic *Arabidopsis* plants. A VirE2–YFP expression cassette was introduced into *Arabidopsis* (ecotype Ws-2) and T2 generation plants were examined by confocal microscopy. **(a)–(d)** Roots; **(e)–(h)** leaf epidermal region; **(i)–(l)** leaf trichome. (a), (e), and (i), DIC images; (b), (f), and (j), YFP fluorescence images; (c), (g), and (k), DAPI fluorescence images showing nuclear staining; (d), (h), and (l), merged YFP and DAPI fluorescence images. Scale bars represent: (a)–(h), 20 μM; (i)–(l), 10 μM. Note the enlarged picture (white box) in Panel D showing perinuclear aggregation of VirE2. Images courtesy of Ms Mei-Jane Fang.

remained in the cytoplasm of transgenic *Arabidopsis* roots cells and in stably transformed tobacco BY-2 cells [15••]. In these latter two studies, the biological activity of VirE2 tagged at its C-terminus was confirmed by the 'complementation' of a *virE2*$^-$ mutant *Agrobacterium* strain by a transgenic plant expressing tagged VirE2. Interestingly, VirE2 tagged at its N-terminus (such as the GUS fusion proteins described above) did not retain biological activity in this assay [15••].

Several recent studies used bimolecular fluorescence complementation to localize interactions between VirE2 molecules or between VirE2 and other proteins. In these experiments, VirE2 was tagged at its C-terminus by a split-YFP molecule (such as the N-terminal region of YFP, nYFP, or the more highly fluorescent derivative nVenus) and allowed to interact with various *Arabidopsis* importin α isoforms tagged with the complementing C-terminal fragment of YFP (cYFP or cCFP). Interaction of VirE2 with most importin α isoforms resulted in restored fluorescence which localized in the cytoplasm, although perinuclear 'rings' of interacting proteins often developed. Only when VirE2 interacted with the importin α isoform IMPa-4 did the proteins colocalize in the nucleus [15••,31]. Interestingly, mutation of only *IMPa4*, but not genes encoding other isoforms of importin α, inhibited *Agrobacterium*-mediated transformation [15••]. These results indicate the special role of IMPa-4 in the transformation process.

VIP1 protein and T-strand nuclear targeting

Resolution of these conflicting subcellular localization results may come from a better understanding of the role played by VIP1 protein in the transformation process. VIP1, which interacts with VirE2, is a b-ZIP category transcription factor normally involved in MAP kinase-mediated plant defense signaling [32]. VIP1 is a phosphoprotein, and the phosphorylation of VIP1 by MPK-3 causes it to relocalize from the cytoplasm to the nucleus [33••]. Nuclear localization of VIP1 is essential for *Agrobacterium*-mediated transformation: coexpression of wild-type VIP1, but not a nonphosphorylatable mutant form of the protein, enhanced transformation of *Arabidopsis* root cell cultures. Thus, differing subcellular localization of VirE2 in various plant cell types (Figure 2) may result from the phosphorylation status of VIP1 in those cells.

Limitations of previous studies involving VirE2 subcellular localization

Additional difficulties in resolving these conflicting VirE2 subcellular localization results and the role of VirE2 in nuclear targeting of T-strands derive both from the techniques used to introduce VirE2 into plant cells and from the different cellular systems employed by the various

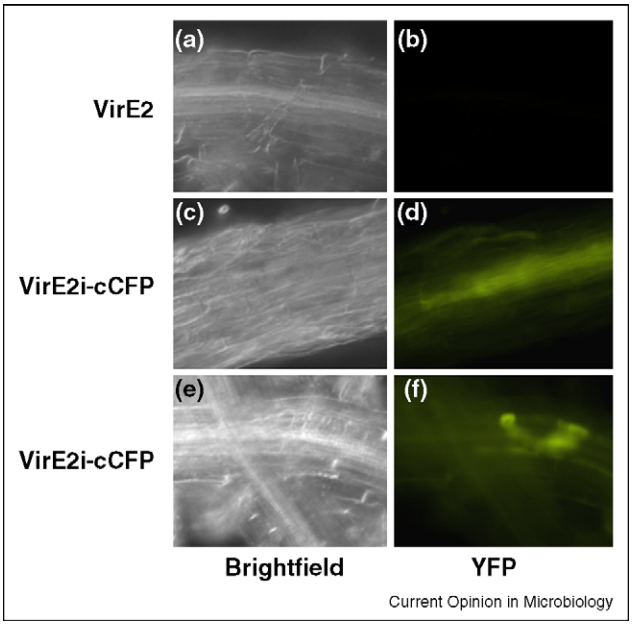
research groups. In all of the studies published to date, large quantities of VirE2 were either introduced into the cells (by microinjection or direct uptake of proteins into permeabilized cells) or synthesized in plants as a result of using strong promoters to drive expression of a VirE2 transgene. Under natural circumstances, however, VirE2 is delivered in much smaller amounts as an effector protein from *Agrobacterium* into plant cells. During *Agrobacterium* infection, VirE2 entry into plant cells requires passage through the plant cellular membrane. *In vitro* black lipid membrane experiments indicate that VirE2 can form a voltage-gated channel for the passage of single-strand DNA [34], and that VirE2 prefers to integrate into membranes whose lipid composition closely resembles that of plant cells [35]. Using optical tweezers, Grange *et al.* [30•] showed that VirE2 could function as a molecular machine to compact cooperatively bound single-strand DNA molecules. These authors hypothesized that after transfer from *Agrobacterium*, VirE2 may temporarily reside in the plant plasma membrane and 'pull' VirD2/T-strands into the plant cell. During this process, VirE2 would coat T-strands and subsequently enter the plant cytoplasm as a VirD2/T-strand/VirE2 T-complex. If this model were correct, the biological validity of many of the experiments described above may be suspect because the experimental approaches bypassed VirE2 entry into the plant cell via the plant plasma membrane.

Novel assays of VirE2 cellular trafficking

In order to resolve the problems of overexpressing VirE2 directly in plant cells, it would be necessary to track VirE2 as it exits *Agrobacterium*, enters the plant cell, and interacts with T-strands and plant proteins. However, this presents a number of biological and technical problems. Because only small amounts of VirE2 protein likely enter the plant cell following infection, it may be necessary to tag VirE2 to increase the sensitivity of detection. However, tagging at the C-terminus would block the T4SS signal and consequently inhibit protein transport from *Agrobacterium* [36–38], and tagging at the N-terminus would block VirE2 function *in planta* [15••]. The author's laboratory has recently found a solution to this problem.

Unlike most of the protein, the N-terminal region of VirE2 is not highly conserved among various *Agrobacterium* strains. Additionally, the N-terminal region can tolerate small insertions of amino acids [39]. We inserted C-terminal fragments of CFP (cCFP, 83 amino acids) individually into several sites within the first 40 amino acids of VirE2 (VirE2i–cCFP). *Agrobacterium* strains mutant in *virE2* but expressing this internally tagged derivative retained virulence, indicating that the insertion of cCFP did not destroy either the transport of VirE2 through the T4SS or VirE2 function *in planta*. We then used 'disarmed' (nontumorigenic) versions of these bacterial strains to infect *Arabidopsis* roots expressing a peptide aptamer fragment of VirE2 corresponding to a known

Figure 3



Transfer of VirE2 from *Agrobacterium* to transgenic *Arabidopsis* roots. *A. tumefaciens* EHA105 (a supervirulent 'disarmed' strain lacking T-DNA) expressing either an untagged VirE2 protein or a VirE2i–cCFP protein was incubated for 40 min with *Arabidopsis* roots expressing a 20 amino acid peptide aptamer, corresponding to a VirE2–VirE2 interaction domain, tagged with nVenus. YFP fluorescence in the roots indicates the transfer of VirE2i–cCFP to plant cells and interaction with the aptamer–nVenus fusion protein. **(a)** and **(b)** Infection using an *A. tumefaciens* strain producing an untagged VirE2 protein; **(c)**–**(f)**, infection using an *A. tumefaciens* strain producing a VirE2i–cCFP protein. (a), (c), and (e), bright field images; (b), (d), and (f), YFP epifluorescence images. Images courtesy of Dr ShouDong Zhang, Purdue University.

VirE2–VirE2 interaction domain. This aptamer was tagged at its C-terminus with the N-terminal fragment of Venus (nVenus). Thus, the roots would fluoresce yellow if VirE2i–cCFP interacted with the aptamer and the N-terminal and C-terminal fragments of the split-YFP derivative fold correctly. Figure 3 shows the results of such an infection, indicating that VirE2i–cCFP had entered the plant cell and interacted with the aptamer peptide. Control experiments using untagged VirE2 did not show yellow fluorescence. Future experiments are planned to look at the subcellular site of interaction of VirE2i–cCFP with the aptamer, and also with VIP1 and IMPa-4 tagged with nVenus.

Alternatives to VirE2

Although most *Agrobacterium* strains encode VirE2 and require this protein for high levels of virulence, some *A. rhizogenes* strains lack both *virE2* and *virE1* [40] (which interacts with VirE2 in *Agrobacterium* and functions as a VirE2 chaperone [41•,42]). Rather, the Ri-plasmids of these strains encode two proteins termed GALLS-FL (full-length GALLS protein) and GALLS-CT (C-terminal region of GALLS protein). GALLS can substitute for

VirE1 and VirE2 in *A. tumefaciens* [43]. Other than the T4SS and NLS signals, GALLS and VirE2 show nothing in common. GALLS contains ATPase and helicase domains not found within VirE2, whereas DNA binding domains found in VirE2 are not conserved in GALLS [44]. GALLS is exported to plants via a T4SS [44]. However, interaction with T-strands has not been reported. Thus, the molecular mechanism by which GALLS mediates transformation in the absence of VirE2 remains unresolved. One hint as to this mechanism derives from the observation that GALLS interacts with VirD2, the protein that caps T-strands [45]. VirE2 also interacts with VirD2 (L-Y Lee, SB Gelvin, unpublished). Ream [46••] has speculated that VirD2 may direct both GALLS and VirE2 proteins to the leading end of T-strands. The resulting interactions would allow T-strands to be 'pulled' into the nucleus, but using different mechanisms. Further investigation of these observations will provide a clearer picture of the role played by VirE2 and GALLS in T-strand nuclear targeting.

Conclusions

Many nucleic acids enter a cell from external sources, whether from bacteria or viruses. For many of these nucleic acids, the final destination is the nucleus where they either replicate or integrate into the host genome. Targeting of nucleic acids to the nucleus involves associated proteins that may derive from the invading organism, the host, or both. When *Agrobacterium* transfers T-DNA to host cells, it also transfers several effector proteins that aid in protecting the DNA and targeting it to the nucleus. These proteins interact with host-encoded proteins to form complexes that guide T-DNA to its final destination, nuclear chromatin. The roles of these proteins, including VirD2, VirE2, and (in the case of certain *A. rhizogenes* strains) GALLS, still require intensive investigation. Knowledge of the mechanism of T-strand nuclear targeting will aid in future efforts to generate transgenic plants from currently recalcitrant crop species, prevent crown gall disease, and understand how alien nucleic acids invade cells.

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