Real-Time Trafficking of *Agrobacterium* Virulence Protein VirE2 Inside Host Cells

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Abstract *A. tumefaciens* delivers T-DNA and virulence proteins, including VirE2, into host plant cells, where T-DNA is proposed to be protected by VirE2 molecules as a nucleoprotein complex (T-complex) and trafficked into the nucleus. VirE2 is a protein that can self-aggregate and contains targeting sequences so that it can efficiently move from outside of a cell to the nucleus. We adopted a split-GFP approach and generated a VirE2-GFP fusion which retains the self-aggregating property and the targeting sequences. The fusion protein is fully functional and can move inside cells in real time in a readily detectable format: fluorescent and unique filamentous aggregates. Upon delivery mediated by the bacterial type IV secretion system (T4SS), VirE2-GFP is internalized into the plant cells via clathrin adaptor complex AP2-mediated endocytosis. Subsequently, VirE2-GFP binds to membrane structures such as the endoplasmic reticulum (ER) and is trafficked within the cell. This enables us to observe the highly dynamic activities of the cell. If a compound, a gene, or a condition affects the cell, the cellular dynamics shown by the VirE2-GFP will be affected and thus readily observed by confocal microscopy. This represents an excellent model to study the delivery and trafficking of an exogenously produced and delivered protein inside a cell in a natural setting in real time. The model may be used to explore the theoretical and applied aspects of natural protein delivery and targeting.

1 Introduction

Agrobacterium tumefaciens can deliver T-DNA into different eukaryotes, including plants (Chilton et al. 1977; Marton et al. 1979; Broothaerts et al. 2005), yeast (Bundock et al. 1995; Piers et al. 1996), algae (Kathiresan et al. 2009), and fungal cells (de Groot et al. 1998). During the transfer process, a single-stranded DNA (T-DNA) molecule is generated inside the bacterium by the VirD1–VirD2 endonuclease (Wang et al. 1984; Yanofsky et al. 1986; Scheiffele et al. 1995). Subsequently, VirD2 remains covalently attached to the 5′ end of the T-DNA (T-strand) (Yanofsky et al. 1986). As a nucleoprotein complex, the T-strand is then transferred into recipient cells via a VirB/VirD4 type IV secretion system (T4SS) (Cascales and Christie 2004) in a manner mechanistically similar to that of a conjugation process (Beijersbergen et al. 1992).

The same T4SS is also known to deliver protein substrates, including VirE2, VirD2, VirE3, VirD5, and VirF (Vergunst et al. 2000; Schrammeijer et al. 2003; Vergunst et al. 2005). These proteins are virulence effectors that interact with host factors in the recipient cells to facilitate transformation. As an abundant Vir protein, VirE2 may coat and protect the T-strand (Citovsky et al. 1988, 1992; Yusibov et al. 1994; Rossi et al. 1996). VirE2 may also mediate uptake of the T-DNA complex by forming a pore in the plant plasma membrane (Dumas et al. 2001). VirE2 could interact with plant VIP1, which is localized in the nucleus upon phosphorylation, and several import in α isoforms in the plant cells, suggesting that VirE2 might

contribute to nuclear targeting of T-DNA (Citovsky et al. 1992; Djamei et al. 2007; Bhattacharjee et al. 2008). In addition, VirE2 might facilitate chromatin targeting of the T-complex through association with host VIP2 (Anand et al. 2007). It appears that VirE2 participates in various steps of T-complex trafficking inside recipient cells, from the entry point to the final destination.

To elucidate how the nucleoprotein complex is trafficked inside recipient cells, it is important to directly visualize the DNA and protein molecules inside the recipient cells after translocation and in real time. A split-GFP system (Cabantous et al. 2005; Pedelacq et al. 2006) was adopted to successfully visualize VirE2, its aggregation forms, and its movement in the recipient cells during a natural transformation process (Li et al. 2014). The split-GFP approach enabled real-time visualization of VirE2 trafficking inside host cells. This system provides a new window to explore the molecular events of *Agrobacterium*-mediated transformation and natural protein delivery processes in real time.

2 Visualization of Delivered VirE2 Protein

2.1 A Split-GFP Approach to Visualize Delivered VirE2

To visualize *Agrobacterium*-delivered VirE2 protein in real time, a split-GFP approach (Cabantous et al. 2005; Pedelacq et al. 2006; Van Engelenburg and Palmer 2010) was adopted so that the delivered VirE2 protein could be detected in a functional form (Li et al. 2014). As shown in Fig. 1A, the split-GFP system is composed of two non-fluorescent GFP fragments: β-strands 1-10 of GFP (GFP1-10) containing 215 amino acid residues (positions 1-215) and β-strand 11 of GFP (GFP11) containing 16 amino acid residues (positions 216-231). GFP1-10 and GFP11 could bind each other spontaneously and restore the fluorescence of GFP_{comp} (Cabantous et al. 2005).

As indicated in Fig. 1, GFP11 was fused onto VirE2 at a permissive site (Zhou and Christie 1999) to create the VirE2-GFP11 fusion. The fusion was expressed inside *A. tumefaciens,* and GFP1-10 was expressed in the recipient cells. When VirE2-GFP11 was delivered into the recipient cells, GFP1-10 would be complemented by VirE2-GFP11 and the resulting VirE2-GFP_{comp} signals were detected (Li et al. 2014).

When GFP11 was fused onto the C-terminus of VirE2, no VirE2-GFP_{comp} signals were detected inside plant cells. This demonstrated that the position of GFP11 tagging was critical for the split-GFP experiments (Li et al. 2014).

Previously, different VirE2 tagging constructs were made to conduct various studies (Bhattacharjee et al. 2008; Aguilar et al. 2010). However, none of the tagged VirE2 proteins could be successfully translocated into recipient cells, presumably because the T4SS channel could not accommodate the enlarged size or a hindering structure of these fusion proteins (Bhattacharjee et al. 2008; Aguilar et al. 2010).

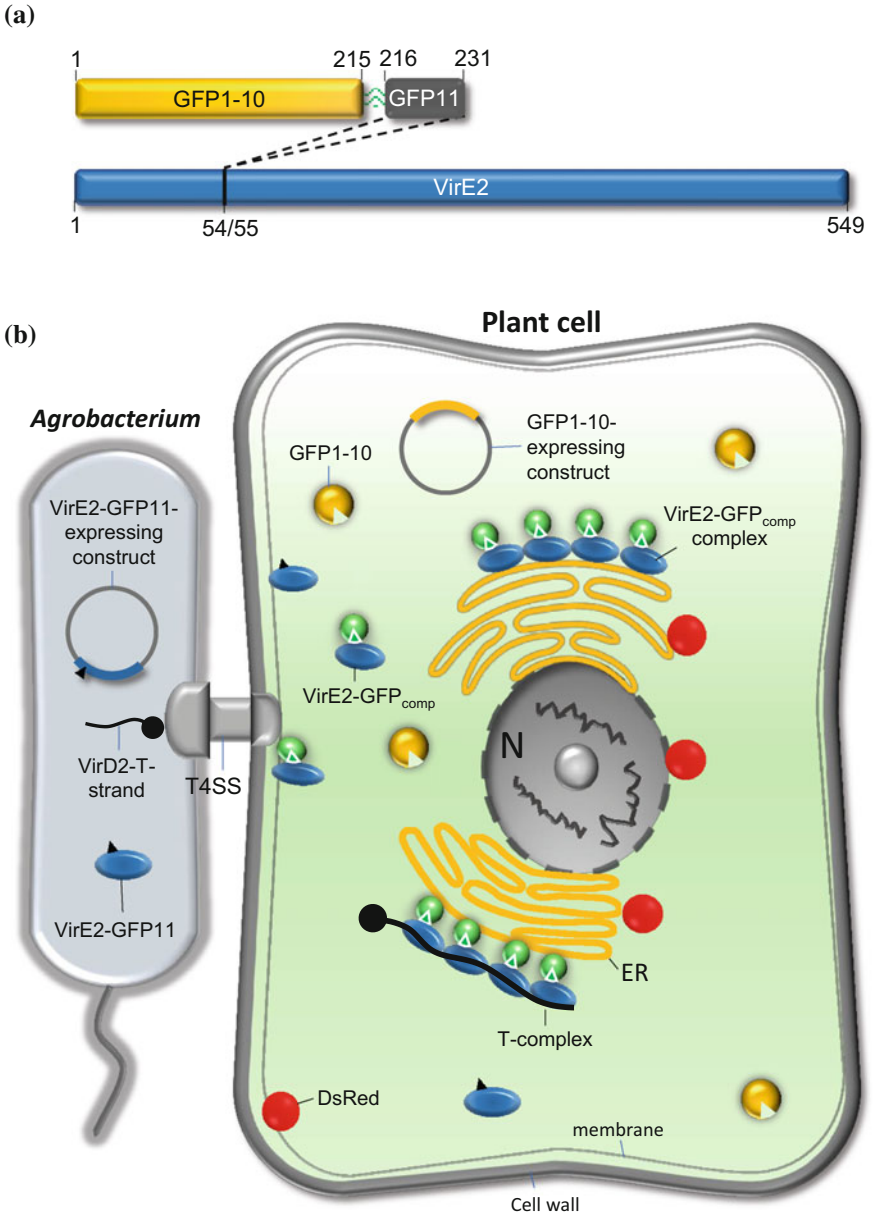

Fig. 1 A split-GFP method to visualize *Agrobacterium*-delivered VirE2 protein inside plant cells. **a** The split-GFP system is composed of two non-fluorescent GFP fragments: β-strands 1-10 of GFP (GFP1-10) containing 215 amino acid residues (positions 1-215) and β-strand 11 of GFP (GFP11) containing 16 amino acid residues (positions 216-231). GFP11 is inserted into VirE2 at amino acid position 54. **b** VirE2-GFP11 fusion is expressed inside *A. tumefaciens*; and GFP1-10 is expressed in the plant cells. When VirE2-GFP11 is delivered into the recipient cells, GFP1-10 would be complemented by VirE2-GFP11 and the resulting VirE2-GFP_{comp} signals are detected. DsRed is expressed inside plant cells to indicate membrane-related structures. Both the T-complex and VirE2-GFP_{comp} complex may be present in the same cell

Nevertheless, the subcellular localization of VirE2 has been investigated with the tagged VirE2 protein. Both cytoplasmic and nuclear localization of VirE2 have been reported (Citovsky et al. 1992; Rhee et al. 2000; Tzfira and Citovsky 2001; Bhattacharjee et al. 2008; Lee et al. 2008; Shi et al. 2014; Lapham et al. 2018). In those studies, VirE2 was artificially introduced into cells either by direct uptake or transgenic expression, which may differ from a natural *Agrobacterium*-mediated transfer process. In addition, VirE2 tagged with a full-length GFP either at the C-terminus or N-terminus may affect its translocation activity (Simone et al. 2001; Atmakuri et al. 2003; Schrammeijer et al. 2003; Bhattacharjee et al. 2008).

The split-GFP approach was also previously adopted to visualize *Agrobacterium*-delivered VirE2, although VirE2 was tagged with GFP11 at the N-terminus (Sakalis et al. 2014). However, N-terminal tagging at VirE2 might affect the role of VirE2 in transformation. Consequently, such a tagged VirE2 might not be suitable to represent the natural VirE2 trafficking inside host cells.

The studies have clearly demonstrated that GFP11 is an appropriate tag that can be fused onto a permissive site of VirE2 so that the VirE2-GFP11 is functional like VirE2 (Li et al. 2014). VirE2-GFP11 movement could represent VirE2 trafficking. Because GFP1-10 was expressed inside recipient cells, the GFP_{comp} signal was only detectable inside recipient cells so that VirE2-GFP11 trafficking signal could be readily monitored.

The monitoring process is straightforward: The bacterial cells expressing VirE2-GFP11 are infiltrated into transgenic *N. benthamiana* (Nb308A) leaves expressing both GFP1-10 and DsRed. The epidermal cells are examined at 2d post-agroinfiltration by confocal microscopy for VirE2-GFP_{comp} and DsRed signals. Images are taken in multiple focal planes (Z-stacks) and then assembled into a movie format. The green fluorescence signals represent VirE2-$GFP_{comp,}$ and the red fluorescence shows the subcellular structures associate with DsRed.

2.2 *Filamentous Structures of Delivered VirE2-GFP*

VirE2 is a non-specific ssDNA-binding protein (Citovsky et al. 1988) that can coat the entire length of T-strands in vitro with one VirE2 molecule covering 19 bases of T-DNA (Citovsky et al. 1997). In the presence or absence of T-DNA, numerous VirE2 molecules can form telephone cord-like multimers in vitro (Citovsky et al. 1997; Frenkiel-Krispin et al. 2007; Dym et al. 2008). This self-association capacity could thus amplify the VirE2-GFP_{comp} signals to facilitate direct visualization of VirE2 inside the recipient cells (Li et al. 2014), as VirE2 is an abundant Vir protein (Engstrom et al. 1987).

When the bacterial cells did not contain T-DNA, the filamentous structures of VirE2-GFP_{comp} signals were also observed, demonstrating that the aggregated form of VirE2-GFP complex could be free of any T-DNA (Li et al. 2014; Yang et al. 2017) (Fig. 1). As VirE2 transfer progressed, more filamentous structures were found and the filaments became even longer. This result suggests that VirE2

aggregation continued when more VirE2 was delivered. Under the same conditions, the negative controls did not generate any GFP fluorescence. These controls included *A. tumefaciens* strains which did not encode VirE2-GFP11 or VirD4. Therefore, naturally transferred VirE2-GFP11 protein and its aggregated form were successfully visualized inside live recipient cells.

2.3 VirE2-GFP11 Functions Similar to VirE2

To ensure that the VirE2-GFP11 movement represents VirE2 trafficking, the VirE2-GFP11 fusion should not disrupt VirE2 function. To achieve this, GFP11 was inserted at Pro54 of VirE2 (Accession No.: AAZ50538) (Li et al. 2014), a site that was shown to be tolerant for a 31-residue oligopeptide insertion (Zhou and Christie 1999). The *virE2* gene from EHA105 was used to generate the VirE2-GFP fusion, which was then utilized to replace the *virE2* gene of EHA105 that does not contain any T-DNA (Hood et al. 1993). Subsequently, VirE2 aggregation and trafficking were studied in the absence of T-DNA.

To test the virulence function of VirE2-GFP, the fusion construct was then used to replace the *virE2* gene of a tumorigenic strain A348 (Li et al. 2014). The resulting A348-*105virE2::GFP11* was inoculated onto roots of transgenic *Arabidopsis thaliana* (H16) expressing GFP1-10. A348-*105virE2::GFP11* caused tumors in a manner similar to the corresponding A348-*105virE2*, which is an A348 derivative with its *virE2* replaced by EHA105 *virE2*. As expected, the *virE2* deletion mutant A348Δ*virE2* was avirulent. The virulence function of VirE2-GFP11 was similar to that of wild-type VirE2, as the frequency and size of tumors caused by A348-*105virE2::GFP11* were similar to those of A348-*virE2*. The results suggest that VirE2-GFP11 was fully functional just like VirE2, even in the presence of GFP1-10 in the transgenic plants. Thus, the VirE2-GFP11 fusion was suitable for visualization of VirE2 and its trafficking upon delivery into recipient cells (Li et al. 2014).

2.4 VirE2-GFP11 Movement Inside Plant Cells

To visualize VirE2 inside a natural-host plant, *A. tumefaciens* EHA105*virE2::GFP11* cells were infiltrated into transgenic *Nicotiana benthamiana* (Nb308A) leaves expressing both GFP1-10 and DsRed. When VirE2-GFP11 was translocated into the plant cells, GFP1-10 bound to VirE2-GFP11 and the resulting VirE2-GFP_{comp} signals appeared as green fluorescence by confocal microscopy (Li et al. 2014). Two days after infiltration, VirE2-GFP_{comp} signals were found in the plant cells in both the cytoplasm and the nucleus. Most of the signals appeared as spots, but some appeared as filamentous structures.

Time course studies indicated that no VirE2-GFP$_{comp}$ signals were detected 16 h after agroinfiltration (Li et al. 2014). At 32 h after agroinfiltration, VirE2-GFP$_{comp}$ signals were detected as spots. At 48 h after agroinfiltration, both VirE2-GFP$_{comp}$ spots and filamentous structures were detected. Pre-induction of the bacteria by acetosyringone (AS) before agroinfiltration did not significantly speed up the appearance or increase the intensity of VirE2-GFP$_{comp}$ signals. This result suggests that *vir* gene induction was not a limiting factor, but it took time for VirE2 to be delivered to a detectable level in plant cells.

VirE2 was suggested to play a role in the T-complex trafficking by hijacking the plant MAPK-targeted VIP1 defense signaling pathway (Tzfira et al. 2002; Djamei et al. 2007). We thus monitored VirE2 movement in a time-lapse series and successfully captured the VirE2-GFP$_{comp}$ trafficking process (Li et al. 2014). The speed of VirE2-GFP movement varied; the majority ranged from 1.3 to 3.1 μm/sec. The movement was nearly linear and directional, suggesting that VirE2 movement was assisted by an active host process.

Both the shorter (dot) and longer (filament) forms of VirE2 aggregation moved inside the plant cells; some movements were directed toward the nucleus (Li et al. 2014). The VirE2-GFP filaments were found to be attached to the nucleus. The filamentous VirE2-GFP complex in the cytoplasm was also found to be linked to the VirE2-GFP complex inside the nucleus. These results suggest that the filamentous VirE2 complex was targeted for nuclear import. When the VirE2 nuclear localization signal 1 (NLS1) (Citovsky et al. 1992) was mutated, the VirE2-GFP complex was exclusively localized in the cytoplasm and nuclear import was not observed either for the VirE2-GFP$_{comp}$ spots or for the filamentous structures (Li et al. 2014). These experiments demonstrated that nuclear import of VirE2-GFP complex was dependent upon the nuclear localization signal.

2.5 *VirE2-GFP11 Does not Move Inside Yeast Cells*

This imaging approach was also applied to the non-natural-host species *Saccharomyces cerevisiae* (Li et al. 2014). A yeast strain encoding GFP1-10 was co-cultivated with *A. tumefaciens* EHA105*virE2::GFP11* that was induced by AS. The VirE2-GFP$_{comp}$ signals could appear as early as 2 h after co-cultivation. However, the signals did not move inside the yeast cells, and they were not localized in the nucleus. More often they were at the periphery of the yeast cells. This indicates that VirE2 is not actively trafficked into the yeast nucleus, presumably because yeast is a non-natural-host recipient and thus lacks the facilitator(s) for VirE2 trafficking. This is consistent with the previous observation that VirE2 was localized in the cytoplasm rather than the nucleus of yeast cells (Rhee et al. 2000).

We found that the transient transformation efficiency of the natural-host plant is 250–500-fold higher than is transformation of the non-natural-host yeast cells, whereas VirE2 delivery was 127–255-fold more efficient than the transient transformation for a non-natural-host recipient (Li et al. 2014). One limiting factor for

the non-natural-host yeast transformation was presumably T-complex trafficking, since the non-natural-host yeast cells could not facilitate active trafficking of the bacterial virulence factors, including VirE2 and perhaps the T-strand. In addition, the filamentous VirE2-GFP structures were not observed inside the yeast cells. It is not clear whether this is due to the limitation of yeast cellular space or the amount of VirE2 delivered into yeast cells.

3 Internalization of Delivered VirE2 Protein into Host Cells

3.1 Delivery Site

To study how VirE2 is internalized into host cells, VirE2 delivery into tobacco cells was observed under a confocal microscope (Li and Pan 2017). The T-DNA-free strain EHA105 was used to avoid any potential complication due to T-DNA trafficking. *A. tumefaciens* EHA105*virE2::GFP11* producing VirE2-GFP11 was infiltrated into transgenic *N. benthamiana* (Nb308A) leaves expressing both GFP1-10 and DsRed. VirE2 delivery into tobacco cells was examined at different time points. A small amount of VirE2 appeared at tobacco cell borders at 32 h after agroinfiltration (Li and Pan 2017). With increasing time, more VirE2 was observed at the cell borders and the VirE2 signals became filamentous. We found that VirE2 first appeared at tobacco cell borders and then moved into the nucleus (Li and Pan 2017).

Subsequently, we determined the spatial positioning of *A. tumefaciens* cells inside plant tissues (Li and Pan 2017). Bacterial cells were constructed to express GFP under the control of the *virB* promoter and thus they became fluorescently labeled naturally during agroinfiltration. After GFP-labeled *A. tumefaciens* cells EHA105 (pAT-GFP) were infiltrated into *N. benthamiana* leaves, most of the bacterial cells were observed to align in the intercellular space of agroinfiltrated tobacco cells (Li and Pan 2017). We observed that the bacterial cells tightly lined up in the intercellular spaces separately as single cells. These results suggest that the limited intercellular spaces of *N. benthamiana* epidermal cells can only accommodate single bacterial cells and the space limitation may allow only the lateral side of the bacterium to closely contact with the host cell.

At 48 h after agroinfiltration, VirE2 accumulated at the cytoplasmic sides of tobacco cells that are in a close contact with *A. tumefaciens* cells (Li and Pan 2017). Interestingly, VirE2 was delivered into plant cells from both sides of the bacterial cells. This observation suggests that a single bacterium could deliver VirE2 into two adjacent host cells simultaneously.

To determine the subcellular location of *Agrobacterium*-delivered VirE2 inside host cells, a specific plant plasma membrane tracker (Nelson et al. 2007) was expressed transiently inside plant cells by T-DNA delivered by the same bacterial cells delivering VirE2-GFP11. *Agrobacterium*-delivered VirE2 appeared to co-localize with the transiently expressed plasma membrane tracker (Li and Pan 2017).

These results suggest that VirE2 is associated with the plant cytoplasmic membrane at the delivery site.

3.2 Mode of VirE2 Internalization

To investigate how membrane-bound VirE2 moved into the cytoplasm, we used a fluorescent styryl dye FM4-64 (Geldner et al. 2003) to label the plant membranes and monitor membrane dynamics (Li and Pan 2017). This lipophilic dye can label membranes where it is applied, but it cannot penetrate the membranes by itself. This property allowed us to monitor the trafficking process of VirE2-bound membranes. *A. tumefaciens* EHA105*virE2::GFP11* cells were infiltrated into *N. benthamiana* leaves to start VirE2 delivery; 48 h later the FM4-64 dye was infiltrated into the same areas. VirE2 co-localized with FM4-64-labeled plasma membranes in a manner similar to that using the plasma membrane tracker. Interestingly, VirE2 co-localized with FM4-64-labeled endomembrane compartments that ranged from 0.8 μm to 4.5 μm in diameter, with an average of 2.2 μm (Li and Pan 2017).

The co-localization of VirE2 with FM4-64-labeled endomembrane compartments continued as FM4-64-labeled vesicles moved inside the cytoplasm (Li and Pan 2017). The speed of movement ranged from 0.4 to 2.1 μm/sec, which is consistent with endosome dynamics as reported in previous studies (Maizel et al. 2011). The data consistently suggest that VirE2 delivered onto host plasma membranes may utilize host endocytosis for cellular internalization and cytoplasmic movement.

3.3 Clathrin Adaptor AP2-Mediated Endocytosis

We examined whether the host endocytosis process was required for internalization of VirE2 protein (Li and Pan 2017). The plant endocytosis process is mediated by clathrin triskelions (McMahon and Boucrot 2011). Overexpression of a C-terminal part of clathrin heavy chain (Hub) that could bind to and deplete clathrin light chains would lead to strong dominant-negative effects on clathrin-mediated endocytosis (CME) (Liu et al. 1995; Kitakura et al. 2011; Dhonukshe et al. 2007).

The effect of Hub overexpression in *N. benthamiana* leaves was then tested and the FM4-64 dye was used to monitor the general endocytosis process. Transient expression of Hub under a CaMV 35S promoter dramatically decreased the internalization of FM4-64 dye (Li and Pan 2017). This result suggests that a dominant-negative strategy using Hub could indeed affect the endocytosis process in *N. benthamiana* epidermal cells. In addition, Hub overexpression increased VirE2 accumulation at cell borders. VirE2 stayed much longer at the cell borders in the tobacco cells overexpressing Hub as compared to the control, indicating that functional clathrin and active CME process were required for VirE2 departure from the plant cellular membrane.

To confirm that host endocytosis is important for VirE2 trafficking, the chemical inhibitor endosidin1 (ES1) was used to interfere with the endocytosis process (Li and Pan 2017), as ES1 affects the endocytosis pathway and causes aggregation of early endosomes in *Arabidopsis thaliana* (Robert et al. 2008). SYP61-mCherry was transiently expressed to label the highly dynamic round-shaped early endosomes (Robert et al. 2008; Foresti and Denecke 2008) in *N. benthamiana* epidermal cells. ES1 treatment caused abnormal VirE2 trafficking within the host cytoplasm; VirE2 accumulated inside the ES1-induced endosome aggregates (Li and Pan 2017). Estimation of co-localization through Pearson's correlation coefficient suggested VirE2 co-localization with the SYP61-mCherry marker, indicating VirE2 co-localization with the early endosomes. These results indicate that ES1 can interfere with host endocytosis and thus restrict VirE2 movement.

Early endosomes mainly function as the sorting hub for endocytic trafficking processes in plants; cargoes internalized from the plasma membrane are usually transported to late endosomes and vacuoles for degradation (Contento and Bassham 2012). To test whether VirE2 is trafficked to late endosomes, ARA6-DsRed (Ueda et al. 2004; Ebine et al. 2011) was transiently expressed to label the late endosomes in *N. benthamiana* epidermal cells (Li and Pan 2017. We did not observe any obvious association of VirE2 with the ARA6-DsRed-labeled late endosome structures (Li and Pan 2017), suggesting that VirE2 may escape from early endosomes and move to other locations to avoid degradation in the vacuoles.

Because *Agrobacterium*-delivered VirE2 was targeted to plant nuclei in a nuclear localization signal (NLS)-dependent manner (Li et al. 2014), we tested the effect of ES1 on nuclear targeting of VirE2 and found that ES1 treatment dramatically decreased the nuclear accumulation of VirE2 inside tobacco cells, while VirE2 accumulated at the cell borders or inside cytoplasm (Li and Pan 2017). This result indicates that ES1 affects VirE2 trafficking rather than delivery or oligomerization of VirE2. These findings suggest that host endocytosis plays an important role in cytoplasmic trafficking and subsequent nuclear targeting of VirE2 inside plant cells.

The importance of endocytosis for transformation was confirmed by studying the effects of chemical inhibitors (Li and Pan 2017). Tumorigenesis assays were conducted using *A. thaliana* roots treated with either ES1 or Tyrphostin A23, which is also a CME inhibitor for *A. thaliana* (Banbury et al. 2003). We found that treatment with ES1 or Tyrphostin A23 significantly attenuated tumorigenesis (Li and Pan 2017). These results suggest that interference with host endocytosis can attenuate the stable transformation of plant cells, presumably because blocked endocytosis affects VirE2 movement.

Endocytosis is a well-conserved process in eukaryotic cells, which is responsible for uptake of a variety of molecules from the outside environment. It participates in a great number of cellular functions such as nutrient uptake, signaling transduction, antigen detection, and cell differentiation (Wu et al. 2014). Although endocytosis is involved in viral entry into host cells (Mercer et al. 2010), it is not clear whether endocytosis is needed for cellular entry of a virulence protein transferred by a bacterial secretion apparatus. These studies demonstrated that *Agrobacterium*

hijacks the host CME pathway for VirE2 internalization into host cells. CME is the major pathway for the endocytosis process (McMahon and Boucrot 2011), which also plays important roles in antigen perception and initiation of plant defense responses upon pathogen infection (Bar and Avni 2014). It would be of interest to determine if *Agrobacterium* might hijack CME-related processes in plant defense to facilitate its infection.

As a highly selective process, recognition and binding of cargo proteins in CME is initiated by clathrin adaptors on the cytoplasmic side of a cell. It is not clear how VirE2 molecules reach the cytoplasmic side of the host cells so that VirE2 can interact with clathrin adaptors. One possibility is that *A. tumefaciens* T4SS can deliver VirE2 directly to the cytoplasmic side of host cells. Alternatively, VirE2 is translocated via additional bacterial or host factor(s) to the cytoplasmic side of host cells. This remains to be addressed.

3.4 VirE2 Internalization Signals

It is of interest to investigate how VirE2 is selected as a cargo for internalization. In general, selection of plasma membrane-associated cargo proteins for internalization depends upon the recognition of endocytic signals at the cytosolic side of cargo proteins by a variety of host adaptors (Bonifacino and Traub 2003; Traub 2009). Upon delivery into host plant cells through a T4SS, VirE2 might interact with a host adaptor protein at the plasma membrane. Sequence analysis indicated that the VirE2 (accession no. AAZ50538) contains five putative endocytic sorting motifs (Li and Pan 2017).

The potential critical leucine or tyrosine residue for each of the dileucine-based or tyrosine-based motifs, respectively, was mutated to alanine; a double mutant was constructed for the two tyrosine-based motifs in the C-terminus (Li and Pan 2017). Neither single mutation nor double mutation of the dual C-terminal tyrosine-based motifs affected VirE2 delivery to the host cellular membrane. However, the double mutation caused a significantly higher level of VirE2 accumulation at the membrane sites. Mutation of other putative endocytic motifs of VirE2 did not affect VirE2 delivery or internalization (Li and Pan 2017). These results suggest that the putative dual C-terminal tyrosine-based motifs are important for VirE2 trafficking.

By conducting assays for transient transformation, we found that both single and double mutations at the dual C-terminal endocytic signals significantly decreased transient transformation efficiency, although the effect of a double mutation (Y488A/Y494A) was more dramatic that was the single mutation Y494A, which affected the function more than Y488A (Li and Pan 2017). These results suggest that the dual C-terminal endocytic signals are required for VirE2 function and that the last endocytic signal at the VirE2 terminus is more important for this function.

Sequence alignment analysis indicated that the dual C-terminal tyrosine-based endocytic motifs are conserved among VirE2 proteins from different Ti plasmids, suggesting their conserved roles in different *Agrobacterium* strains (Li and

Pan 2017). Moreover, mutation of these conserved motifs of VirE2 from the virulent strain A348 also attenuated tumor formation on *Arabidopsis* root fragments (Li and Pan 2017). These results demonstrate that the dual tyrosine-based endocytic signals located at the VirE2 C-terminus are important for VirE2 function for both transient and stable transformation.

3.5 VirE2 Internalization Signals Interact with Plant AP2M

We hypothesize that the dual C-terminal tyrosine-based endocytic motifs of VirE2 might be recognized by a clathrin-associated sorting protein (Li and Pan 2017). The clathrin-mediated endocytosis process is facilitated by a group of host adaptors known as "clathrin-associated sorting proteins," which are responsible for endocytic signal recognition and cargo binding (Bonifacino and Traub 2003; Traub 2009). Among them, the adaptor protein 2 (AP-2) complex recognizes the tyrosine-based endocytic signal and binds to it through the C-terminal domain of the μ-subunit (AP2M) (Jackson et al. 2010).

When the C-terminal tail was fused to GST (GST-VirE2C), VirE2 interacted with the cargo-binding domain of AP2M that was fused to the maltose binding protein (MBP; MBP-AP2MC) (Li and Pan 2017). However, a double mutation at the dual tyrosine-based endocytic signals eliminated this interaction. These results suggest that AP2M recognizes and binds to the VirE2 C-terminal tail through the dual tyrosine-based sorting motifs.

The importance of the host AP-2 complex in transformation was confirmed by testing two insertional mutants of *A. thaliana* AP2M for tumorigenesis (Li and Pan 2017). These two mutants have been previously shown to display defects in endocytosis (Kim et al. 2013). We found that these mutants of AP2M displayed significantly attenuated tumor formation, as compared to the wild-type control (Li and Pan 2017). These results demonstrate that the host AP-2 complex is important for *Agrobacterium*-mediated transformation of plant cells.

Membrane-associated protein cargos are internalized to form clathrin-coated vesicles (CCVs) and are then transported to the other parts of the cell (Bonifacino and Traub 2003; Traub 2009). The AP-2 adaptor complex is composed of two large subunits (α and β2), one medium-sized subunit (μ2), and one small subunit (σ2); it specifically recognizes and binds to the tyrosine-based (YXXØ) and dileucine-based ([DE]XXXL[LI]) sorting motifs on cargo proteins (Traub and Bonifacino 2013). We demonstrated that two closely located tyrosine-based motifs on the VirE2 C-terminus were responsible for VirE2 interaction with the cargo-binding domain of μ subunit of the AP-2 adaptor complex (Li and Pan 2017). Mutation of these signal sequences decreased VirE2 internalization and impaired transformation, suggesting that the dual tyrosine-based motifs are important for VirE2 trafficking and function during AMT. The second motif appeared to play a more important role in the transformation process. These two motifs might have different binding affinity and accessibility to the AP-2 complex.

Previous studies showed that the AP-2 complex is responsible for cargo transportation between the plasma membrane and early endosomes in plant cells (McMahon and Boucrot 2011; Chen et al. 2011). Our data suggest that interaction of VirE2 with the AP-2 complex can facilitate VirE2 internalization from the host plasma membrane (Li and Pan 2017). However, the root transformation assays demonstrated that mutations at the critical dual motifs decreased the efficiency of transformation by only 30-fold (Li and Pan 2017), whereas VirE2-deletion mutants virtually did not generate any transformants (Li et al. 2014). These results suggest that VirE2 may also be trafficked via an alternative pathway(s) for its role in the transformation process.

We observed that the speed of VirE2 movement together with endomembrane compartments ranged from 0.4 to 2.1 μm/sec (Li and Pan 2017). After VirE2 departs from the host membranes via endomembrane compartments, VirE2 would be trafficked to subsequent locations. Early endosomes serve as the main sorting hub for both secretory and endocytic trafficking cargoes. Different pathogens and viruses have been reported to use host endocytic pathway to facilitate infection (Gruenberg and van der Goot 2006; Cossart and Helenius 2014). Pathogen effectors and viruses have evolved and developed a variety of approaches to escape from host endosomes after internalization (Spooner et al. 2006; Personnic et al. 2016). To reach the host nucleus, VirE2 would need to escape from endosomes to avoid degradation in the host cytoplasm. This is indeed supported by our observation that VirE2 was not associated with plant late endosomes (Li and Pan 2017). Thus, further studies are needed to investigate how VirE2 escapes from endosomes and moves to other parts of host cells.

4 Trafficking of Delivered VirE2 Protein Inside Host Cells

4.1 VirE2 Moves Along the ER

To determine the cellular structure facilitating VirE2 trafficking, we conducted double labeling experiments (Yang et al. 2017). *A. tumefaciens* cells EHA105*virE2::GFP11*, encoding a VirE2-GFP11 fusion, were infiltrated into the leaf tissues of transgenic tobacco (Nb308A) plants, which constitutively expressed GFP1-10 and free DsRed, which labeled the cellular structures and nucleus (Li et al. 2014). Upon delivery into plant cells by the bacterium, VirE2-GFP11 complemented GFP1-10. The resulting VirE2-GFP_{comp} signals started to appear inside plant cells 2d after agroinfiltration.

The VirE2-GFP_{comp} signals moved along a strand-like cellular structure labeled with free DsRed (Yang et al. 2017). Interestingly, direct entry of VirE2 into the nucleus was visualized. VirE2 moved faster along linear tracks, although the velocities varied on different linear tracks and moved slower along curved tracks.

Chemical inhibitors were used to study the potential cellular structure that might facilitate the movement of *Agrobacterium*-delivered VirE2 inside plant cells

(Yang et al. 2017). Cytochalasin D (CytoD) and brefeldin A (BFA) had a significant effect on VirE2 trafficking, whereas colchicine (Colc) had only a minor effect.

CytoD is a potent inhibitor of actin polymerization (Krucker et al. 2000). BFA inhibits protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus by dilating the ER (Misumi et al. 1986). Colc inhibits microtubule polymerization (Skoufias and Wilson 1992). The effects of these inhibitors on the corresponding cellular structures were observed in tobacco leaves. Colc, BFA, and CytoD disrupted the microtubules, ER, and actin structures, respectively. Therefore, we hypothesized that VirE2 trafficking was facilitated by ER/actin structures (Yang et al. 2017).

We determined if VirE2 movement is associated with the ER (Yang et al. 2017) by using an ER-mCherry construct containing an ER-targeting sequence at the N-terminus and the tetrapeptide retrieval signal HDEL at the C-terminus (Nelson et al. 2007). We found that VirE2 aggregates appeared as dots and filaments inside tobacco cells. Both forms were co-localized with inter-connected ER tubules that were shown by ER-mCherry. Time-lapse imaging showed that VirE2 aggregates moved along the ER strands (Yang et al. 2017).

As ER membranes compartmentalize the intracellular space into ER lumen and cytosol, it was necessary to determine whether VirE2 was present on the cytosolic or luminal side. By using an ER-targeting sequence and conducting the split-GFP complementation experiments, we demonstrated that *Agrobacterium*-delivered VirE2 was on the cytosolic side of the ER after delivery into the plant cytoplasm (Yang et al. 2017).

4.2 VirE2 Moves on an ER/F-Actin Network

We determined whether VirE2 movement is associated with F-actin filaments (Yang et al. 2017). To visualize both F-actin and VirE2, an F-actin marker tdTomato-ABD2 (Nakano et al. 2009) was expressed by agroinfiltrating EHA105*virE2::GFP11* into transgenic *N. benthamiana* Nb307A leaves expressing GFP1-10. We found that VirE2-GFP_{comp} signals co-localized with F-actin filaments. Time-lapse imaging demonstrated that VirE2 moved along an F-actin filament (Yang et al. 2017).

VirE2 affects the fate of T-DNA in many ways (Ward and Zambryski 2001). Therefore, it is important to determine how VirE2 is trafficked through the cytoplasm and reaches the nucleus. VirE2 contains two bipartite NLS signals (Citovsky et al. 1992), which are present on the exterior side of the solenoidal structure (Dym et al. 2008). This structural arrangement may make the NLS signals available to interact with other host factors. When the NLS of VirE2 was mutated to be recognizable in animal cells, the "animalized" VirE2 was found to migrate along microtubules in cell-free *Xenopus* oocyte extracts, propelled by dynein motors (Salman et al. 2005). However, no plant dyneins have been found (Lawrence et al. 2001). It remains unknown whether VirE2 moves along microtubules in plant cells.

Therefore, the trafficking of "animalized" VirE2 in animal cells may not accurately represent the mechanism of VirE2 trafficking inside plant cells. Moreover, disruption of microtubules by colchicine did not affect VirE2 trafficking significantly (Yang et al. 2017). These results suggest that VirE2 uses a transport system other than microtubules when trafficked inside plant cells. Our study showed that *Agrobacterium*-delivered VirE2 was trafficked via the ER and F-actin network (Yang et al. 2017). The VirE2-associated T-complex may also use the same trafficking mode, as VirE2 can coat the surface of the T-complex.

We hypothesize that *Agrobacterium* has evolved to enable VirE2 to exploit ER streaming, which is part of the cytoplasmic streaming process. *Agrobacterium*-delivered VirE2 is associated with the ER (Yang et al. 2017), probably because of the high affinity of VirE2 for membranes (Dumas et al. 2001). However, it is possible that an unknown factor(s) is responsible for VirE2-ER association. The VirE2-associated ER might be thus driven primarily by the ER-associated myosin XI-K. The myosin-associated ER can move along actin filaments. Therefore, *Agrobacterium*-delivered VirE2 might be trafficked through plant cells via the myosin-powered ER/actin network, because of the dynamic three-way interactions between the ER, F-actin, and myosin (Ueda et al. 2010).

The ER stretches through the entire cytoplasm and continues to the outer membrane of the nucleus, which may provide VirE2 with a convenient path to reach the nucleus. Cytosolic facing of VirE2 on the ER seems to make the opening of the nuclear pore complex accessible for nuclear import of VirE2. Association of VirE2 with the ER also suggests that VirE2 may interact with other factors during the trafficking processes. Indeed, a SNARE-like protein was found to have a strong interaction with VirE2 (Lee et al. 2012). It has also been reported that reticulon domain proteins and a Rab GTPase, both involved in trafficking of proteins through endomembranes, are important for transformation (Hwang and Gelvin 2004). These findings suggest that vesicular budding or fusion processes may be involved in VirE2 trafficking inside the cytoplasm.

4.3 VirE2 Movement Is Powered by Myosin

The speed of VirE2-GFP movement ranged from 1.3 to 3.1 μm/sec, and the movement was linear and directional (Yang et al. 2017). These results suggest that VirE2 trafficking inside plant cells is an active process that may be powered by myosin.

We determined whether myosin plays a role in VirE2 movement inside plant cells (Yang et al. 2017), as ER/F-actin/myosins may exhibit a three-way interaction (Ueda et al. 2010). A selective myosin light-chain kinase inhibitor ML-7 (Saitoh et al. 1987) was used to inhibit plant myosin activity. Treatment with ML-7 inhibited VirE2 movement as the average velocity was reduced by 95% relative to that of the control.

A dominant-negative approach was adopted to identify the specific myosin responsible for VirE2 movement (Yang et al. 2017). Several dominant-negative mutants of plant myosin genes were overexpressed during *Agrobacterium*-mediated delivery of VirE2. *A. tumefaciens* cells containing T-DNA encoding the tail constructs were co-infiltrated with EHA105*virE2::GFP11* into tobacco (Nb308A) plants. The myosin tail expression took place later than VirE2 delivery, so that the myosin mutant constructs would not affect VirE2 delivery. Among the myosin mutants tested, only the XI-K tail inhibited VirE2 trafficking (Yang et al. 2017). These data suggest that myosins provide the driving force for VirE2 movement, and that myosin XI-K is the most important contributor.

To determine if the VirE2 movement observed during our study was directly related to *Agrobacterium*-mediated transformation, the effect of the selective myosin light-chain kinase inhibitor ML-7 was tested on *Arabidopsis* root transformation. We found that ML-7 significantly reduced the transformation efficiency (Yang et al. 2017), while ML-7 did not affect the growth of root segments or *Agrobacterium* growth. These results suggest that the inhibition of myosin activity might have reduced the transformation efficiency.

To confirm the specific effect of myosin inhibition on transformation, RNAi constructs containing a partial sequence of XI-2 and XI-K (Avisar et al. 2008) were used to silence the corresponding genes (Yang et al. 2017). The RNAi constructs used for these experiments generated specific but not off-target effects (Avisar et al. 2008). We found that silencing of XI-K attenuated tumor formation (Yang et al. 2017). These data clearly indicate that XI-K affects VirE2 movement, and thereby *Agrobacterium*-mediated transformation.

Our study also showed that VirE2 trafficking may require the plant-specific myosin XI family and XI-K in particular (Yang et al. 2017). Myosin XI family members are involved in cytoplasmic streaming (Yokota et al. 1999), ER motility (Ueda et al. 2010), and trafficking of organelles and vesicles (Avisar et al. 2008). Despite the conformational similarities with myosin V, myosin XI has a plant-specific binding mechanism (Li and Nebenführ 2007) and thus recognizes different cargos than myosin V. This may provide an explanation for the very significant difference in transformation efficiency between yeast and plant recipients (0.2% in *S. cerevisiae* vs. 100.0% in *N. benthamiana*). The efficiency of protein delivery is comparable between yeast and plants (50.9% in *S. cerevisiae* vs. 100.0% in *N. benthamiana*) (Li et al. 2014). The budding yeast *S. cerevisiae* lacks myosin XI-K, which would render VirE2 immobile in the yeast cells. Thus, the transformation efficiency is significantly reduced. Our study demonstrated that myosin XI-K plays a much more critical role in VirE2 trafficking than does XI-2 (Yang et al. 2017). Both XI-K and XI-2 are highly expressed inside plant cells (Peremyslov et al. 2011). However, myosin XI-K is the primary contributor to ER streaming (Ueda et al. 2010).

4.4 Trafficking Mode of Delivered VirE2

The studies demonstrated that VirE2 is trafficked inside plant cytoplasm via a myosin XI-K-powered ER/actin network (Yang et al. 2017). This may indicate how the T-complex is trafficked inside host cells, because its surface consists of VirE2 molecules. VirE2 was visualized in real time to be trafficked toward the plant nucleus along a linear cellular structure that was illustrated by free DsRed molecules. This linear structure was later determined to be part of the ER/actin network, based on experiments using chemical treatments and fluorescent marker labeling. Moreover, VirE2 was present on the cytosolic side of the ER. Myosin XI-K provided the driving force for VirE2 movement (Yang et al. 2017).

The presence of VirE2 on the cytoplasmic side of the ER enables us to speculate about the trafficking mode of delivered VirE2 protein. As the ER is linked to the outer membrane of the nuclear envelope, delivered VirE2 should reside on the same topological surface as the nuclear opening. Because the ER is a dynamic structure that is involved in continuous flow and movement of lipids and proteins, the associated VirE2 may move along the ER inside the cytoplasm. This might explain why VirE2 can readily reach the nuclear opening for efficient nuclear import.

As shown in Fig. 2, we hypothesize how myosin XI-K powers VirE2 trafficking. One possibility is that myosin XI-K recognizes VirE2 with its globular domains and drives its movement, although there is no experimental evidence for this interaction yet (Yang et al. 2018). Another possibility is that ER-associated VirE2 may be present in a vesicle form that may be recognized by myosin XI-K (Yang et al. 2018). In this model, myosin XI-K indirectly drives VirE2 movement by carrying VirE2-containing cargoes; indeed, some myosin XI-K-specific vesicle adaptors have been identified (Kurth et al. 2017) although it is not clear whether VirE2 is associated with such a vesicle. Alternatively, VirE2 might passively follow the flow of the ER because of its association with the ER, although VirE2 and myosin XI-K might not have any interactions (Yang et al. 2018).

5 A Natural Model to Study Exogenous Protein Delivery

Delivery of materials into cells is a critical component of genetic engineering, genome-editing, therapies, and a diversity of fundamental research applications. However, efficient intracellular delivery of exogenous compounds and macromolecular cargo remains a long-standing challenge. The limitations of established delivery technologies have hampered progress in multiple areas, as the potential of exciting new materials, insights into disease mechanism, and approaches to cell therapy have not been fully realized because of the delivery hurdles. There is an urgent need to develop next-generation approaches for intracellular delivery (Delvigne et al. 2015), which are safe and efficient.

The natural molecular tracking system VirE2-GFP (Li et al. 2014; Li and Pan 2017; Yang et al. 2017) may be particularly useful for studying protein delivery and

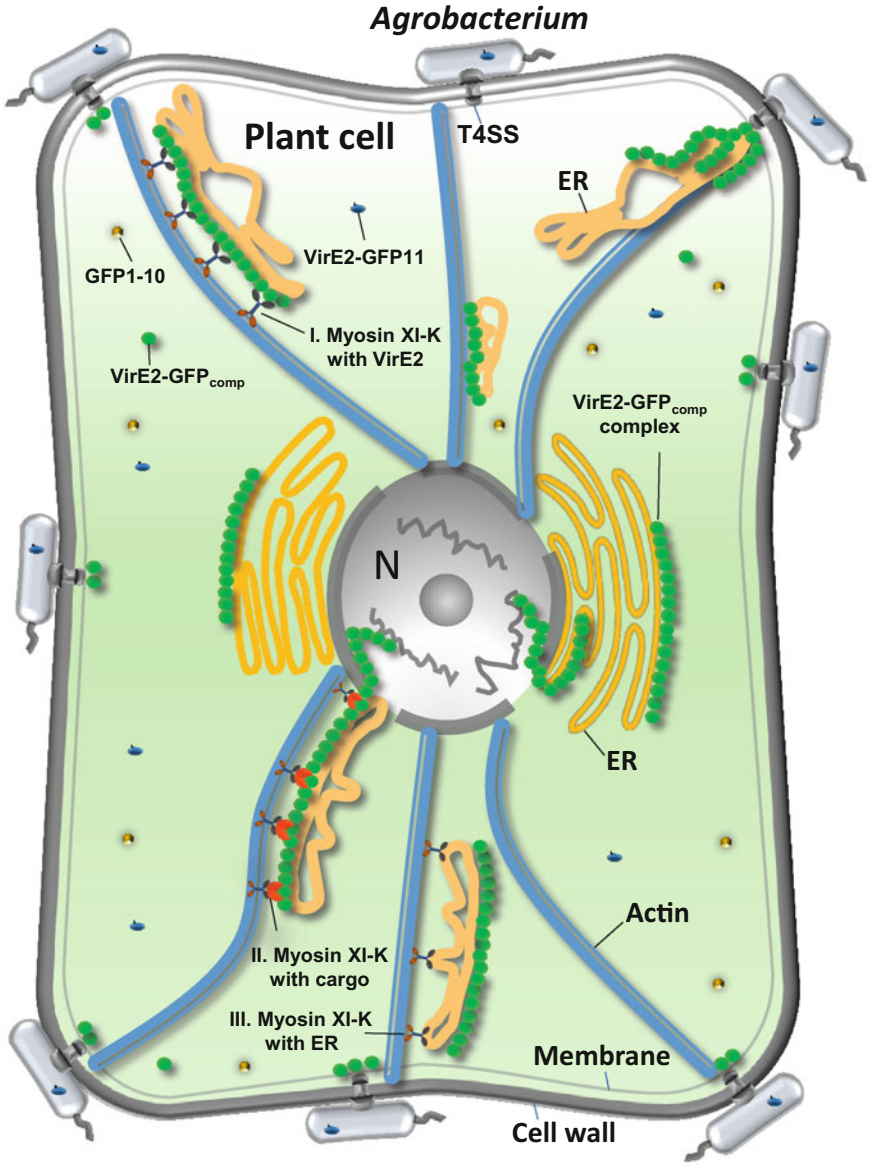

Fig. 2 A natural system to visualize the protein delivery process. VirE2-GFP11 fusion is expressed inside *A. tumefaciens*; and GFP1-10 is expressed in the plant cells. When VirE2-GFP11 is delivered into the plant cells, GFP1-10 would be complemented by VirE2-GFP11 and the resulting VirE2-GFP_{comp} signals can be detected. As VirE2-GFP11 is functional like VirE2, VirE2-GFP11 movement could represent the VirE2 trafficking. VirE2-GFP_{comp} complex starts to appear at the VirE2 delivery site and moves toward the nucleus. The movement is powered by myosin XI-K in three possible ways: (i) myosin XI-K interacts directly with VirE2; (ii) myosin XI-K interacts with a cargo associate with VirE2; and (iii) myosin XI-K interacts with ER associated with VirE2

trafficking (Fig. 2). VirE2 is a natural protein that can self-aggregate and contains the targeting sequences so that it can efficiently move all the way from the bacteria to the host plant nucleus. We adopted a split-GFP approach and generated the VirE2-GFP fusion (Li et al. 2014), which retains the self-aggregating property and the targeting sequences. The fusion protein is fully functional; it can be produced inside the bacterium and then delivered into plant cells and trafficked toward the plant nucleus in a readily detectable format: fluorescent and unique filamentous aggregates (Li et al. 2014). VirE2-GFP can track molecular events inside a cell in a natural setting in real time (Li et al. 2014). It is convenient to conduct experiments with this approach, as a large amount of VirE2-GFP can be delivered into the cells and the efficiency of delivery can be up to 100% of recipient cells that are in close contact with the bacterial cells (Li et al. 2014). This is useful to obtain more accurate insight into the delivery process.

Using the GFP-VirE2 system, we discovered that the exogenously produced protein VirE2 is naturally delivered into plant cells via clathrin-mediated endocytosis. We identified a VirE2 internalization signal that can target VirE2 (Li and Pan 2017) and potentially other macromolecules into cells. We also found that the delivered VirE2 is subsequently trafficked inside plant cells via a myosin XI-K-powered ER/actin network (Yang et al. 2017). We found that the VirE2-GFP system can be used as a new method to test drug toxicity, as described below. These findings demonstrate the usefulness of the VirE2-GFP-based imaging approach to study protein delivery and sorting.

6 A Test System to Examine the Effect of a Molecule on Cells

A biological activity is often studied in an unnatural setting, such as an in vitro system, and in a snapshot manner, for instance, by measuring a reporter activity at different time points. Furthermore, current reporters normally focus on one aspect of the biological process at a time; they are not suitable to study the overall wellbeing of the cell. These systems may therefore generate inaccurate conclusions and undesirable products.

The natural molecular tracking system VirE2-GFP11 may be defined as a bio-tracker, as the fusion protein is fully functional and can move inside cells in a readily detectable format: fluorescent and unique filamentous aggregates (Fig. 2). It is much easier to recognize the VirE2-GFP signals than GFP reporter activity. Importantly, VirE2-GFP can bind to membrane structures such as the endoplasmic reticulum (ER) that are trafficking inside a cell. This enables us to readily observe the highly dynamic activities of the cell. If a compound, a gene, or a condition affects the cell, the cellular dynamics shown by VirE2-GFP will be affected and thus readily observed under confocal microscopy. We found that the assay is highly sensitive and can readily detect minor effects on the cell.

Thus, VirE2-GFP can be used as a new research tool to label a cell, tissue, organ, organ system, or organism. This can facilitate real-time studies on the cell, tissue, organ, organ system, or organism in a natural setting. It enables the researcher to address new questions that cannot be addressed by current approaches.

The technology can study the wellbeing of a cell in a sensitive, rapid, and visually understandable manner. This will help improve the accuracy of biological research and develop quality products and services to meet pressing challenges. The bio-tracker-based toxicity testing method provides a new approach, which not only can reduce the use of animals, but also can present the toxicity in a visually understandable format. This is useful to improve the relations between the public and drug development.

As an example, we conduct non-specific toxicity tests as follows.

A. tumefaciens cells EHA105*virE2::GFP11*, encoding VirE2-GFP11 fusion, were infiltrated into the leaf tissues of transgenic tobacco (Nb308A) plants, which constitutively express GFP1-10 and free DsRed to indicate the cellular structures and the nucleus. Transgenic tobacco (Nb308A) plants were treated at 42 h post-agroinfiltration with the chemicals econazole and ketoconazole. The effects were observed 6 h later. As shown in Fig. 3, the effects of econazole and

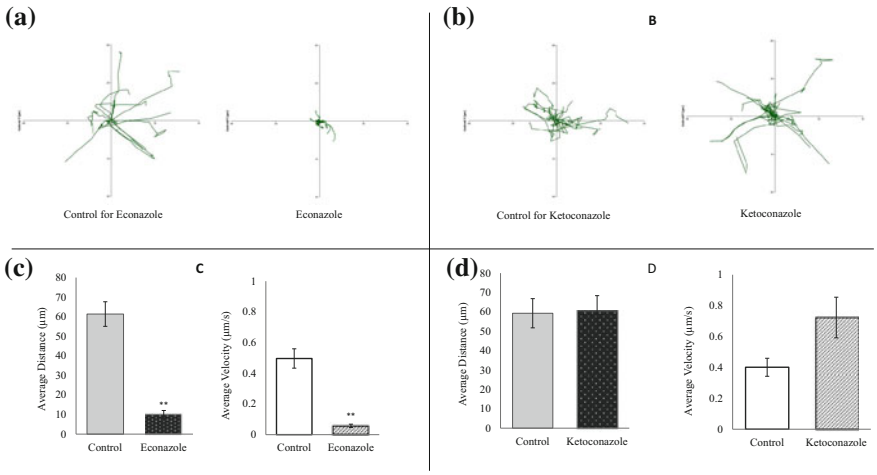

Fig. 3 Comparison of the non-specific toxicity between econazole and ketoconazole. *Agrobacterium* strain EHA105-VirE2::GFP11 was infiltrated into the leaves of *N. benthamiana* transgenic plant Nb308A. Chemical infiltration was performed 42 h after agroinfiltration. The control groups were set by infiltration with only the respective solvent aqueous dilutes. Six hours after chemical infiltration, images were taken in multiple focal planes (Z-stacks) with a step interval of 1.0 μm using a spinning disk confocal microscope system with Olympus UPLSAPO 60×/1.20 water. Time-lapse imaging was set at 3 min. The movement of VirE2 signals was tracked with "manual tracking" function provided by Volocity 3D Image Analysis Software (Ver. 6.2.1). Tracking plots, track lengths, and velocities were generated automatically after tracking was finished. Tracking plots (**a** and **b**) are presented in such a way that very movement track is assumed to start at the same origin. Mean track velocity and mean displacement of VirE2 movement (**c** and **d**) were measured; the data analysis was performed using ANOVA (*$P < 0.05$; **$P < 0.01$). The effects of econazole (**a** and **c**) at 45 μM and ketoconazole (**b** and **d**) at 94 μM were tested

ketoconazole on VirE2-GFP movement can be readily differentiated. Although both of them are imidazole derivatives and known to fight fungal infections, econazole caused much more toxicity effects on the plant cells than did ketoconazole, based on the pattern, distance and velocity of VirE2-GFP movement pattern. This is consistent with the fact that econazole indeed causes more harm in humans than does ketoconazole.

Because plant cells are very much different from the human cells, we consider the effect on VirE2-GFP movement as non-specific toxicity. Using plant cells to test non-specific toxicity should represent a new approach to assess the non-specific toxicity of a drug or compound.

7 Conclusions

Agrobacterium is widely used as a genetic vector to deliver DNA into various cells, whereas its capacity to deliver proteins is not fully explored. In a sense, *Agrobacterium* is regarded as a genetic engineer and not widely used as a vector for protein delivery. Direct visualization of *Agrobacterium*-delivered VirE2 protein, based on a split-GFP approach, indicated that *Agrobacterium* is more efficient in protein delivery than genetic transformation for a non-natural-host recipient. It should be of significance to further explore the capacity of *A. tumefaciens* to deliver proteins.

Visualization of VirE2 inside recipient cells may be also useful to study the trafficking pathway of T-strands, as VirE2 is a component of the proposed nucleoprotein complex. The abundance of VirE2 (Engstrom et al. 1987) is particularly suitable for its role to protect T-DNA by coating it with numerous molecules (Citovsky et al. 1988). It was estimated that about every 19 bases of T-DNA is coupled with one VirE2 molecule (Citovsky et al. 1997). In addition, VirE2 protein can also assemble without T-DNA to form homodimers and solenoids (Frenkiel-Krispin et al. 2007). These unique traits prompted us to use VirE2 as a model to study *Agrobacterium*-delivered molecules inside recipient cells.

The split-GFP approach enabled us to directly visualize *Agrobacterium*-delivered VirE2 in live recipient cells. Because VirE2-GFP11 is delivered in a natural setting, VirE2-GFP11 movement should represent the natural trafficking process inside recipient cells. This should be of use to further study how host factors facilitate VirE2 movement inside recipient cells.

As a natural genetic engineer, *A. tumefaciens* can cause crown gall disease on an exceptionally wide range of host plants in nature (De Cleene and De Ley 1976). The bacterium can achieve a high efficiency of transformation that can approach 100% (Li et al. 2014). Using VirE2-GFP as the model, we show that *Agrobacterium* uses the host endocytic process to facilitate delivery and trafficking for one of its virulence factors, VirE2, into host cells. Endocytosis is a well-conserved fundamental process in all eukaryotic cells. It may occur independently of cell types or differentiation. In addition, the bacterium hijacks a

conserved host network to move virulence factor VirE2 toward the nucleus. These may be important for *Agrobacterium* to achieve both a wide host range and a high efficiency.

VirE2-GFP may be used as a bio-tracker, because the VirE2-GFP fusion retains the self-aggregating property and the targeting sequences. In addition, the fusion protein is fully functional and can move inside cells in a readily detectable format: fluorescent and unique filamentous aggregates. VirE2-GFP can bind to some membrane structures such as the endoplasmic reticulum (ER) that are trafficking inside a cell. This enables us to readily observe the highly dynamic activities of the cell. If a compound, a gene, or a condition affects the cell, the cellular dynamics shown by the VirE2-GFP may be affected and thus readily observed under confocal microscopy. We found that the assay is highly sensitive and can readily detect minor effects on the cell.

Thus, VirE2-GFP can be used as a new research tool to label cells, tissues, organs, organ systems, or organisms. This system can facilitate real-time studies on the cell, tissue, organ, organ system, or organism in a natural setting. It enables the researcher to address new questions that cannot be addressed with the current approaches.

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