RESEARCH ARTICLE

Direct fluorescence detection of VirE2 secretion by Agrobacterium tumefaciens

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Abstract

VirE2 is a ssDNA binding protein essential for virulence in Agrobacterium tumefaciens. A tetracysteine mutant (VirE2-TC) was prepared for in vitro and in vivo fluorescence imaging based on the ReAsH reagent. VirE2-TC was found to be biochemically active as it binds both ssDNA and the acidic secretion chaperone VirE1. It was also biologically functional in complementing virE2 null strains transforming Arabidopsis thaliana roots and Nicotiana tabacum leaves. In vitro experiments demonstrated a two-color fluorescent complex using VirE2-TC/ReAsH and Alexa Fluor 488 labeled ssDNA. In vivo, fluorescent VirE2-TC/ReAsH was detected in bacteria and in plant cells at time frames relevant to transformation.

Importance

Cell to cell transfer of proteins and nucleic acids lies at the heart of many biological processes. Detecting such transfer is often problematic or indirect. We show here that the biaresenical fluorescent reagent ReAsH can be used to reveal the presence of the secreted effector VirE2 from Agrobacterium tumefaciens in the cytoplasm of host plant cells. Our studies establish a new method for monitoring translocation of the VirE2 effector from A. tumefaciens to plant target cells during the infection process.

Introduction

Agrobacterium tumefaciens is a Gram negative soil pathogen of plants. It has the ability to transform dicotyledonous plants, leading to formation of tumors known as “crown galls”. This disease results from the transfer of a single strand DNA segment from the large tumor inducing (Ti) plasmid carried by the bacterium into the plant host cell [1]. Based on a type IV secretion system, the transfer process is similar to bacterial conjugation. Virulence genes (vir) responsible for the DNA transfer reside on the same Ti-plasmid as the transferred DNA (T-DNA). These encode for protein components of the secretion system itself, for the processing machinery to excise a single strand of T-DNA and bind a mobility protein (VirD2) to the 5’ end, and for accompanying effector proteins [2]. The most abundant of these is VirE2, a 63.5 kDa ssDNA-binding protein that is essential for efficient transformation [3–5].
vitrō, VirE2 binds the T-strand cooperatively, without sequence specificity. The resulting T-complex is a solenoidal structure that has been extensively characterized [6–9]. In planta, VirE2 protects the T-strand from degradation [10], though evidence for a direct interaction is still lacking. VirE2 possesses nuclear localization signals [11–13] and interacts with a specific importin-α isoform, IMP-α4 [14], as well as with the host plant transcription factor VIP1 [15,16].

In evidence for an interaction in the host cell, the T-strand and VirE2 protein may be transported to it separately. Extracellular complementation assays showed that coinoculation of plant with a combination of two Agrobacterium strains, one lacking T-DNA but containing VirE2, the other lacking VirE2 but containing T-DNA, leads to successful plant transformation [17]. Moreover, VirE2 expressed exogenously in plant cells binds T-strand delivered from A. tumefaciens to mediate formation of tumors, even if the infecting bacterial strain lacks VirE2 [11]. Nonetheless the parameters governing encounter between VirE2 and the T-strand remain unknown when these do originate, as naturally, from the same bacterium.

In Agrobacterium tumefaciens, VirE2 is expressed together with VirE1, a small acidic chaperone that is essential for VirE2 secretion and pathogenicity [18,19]. VirE2 expressed in vitro in the absence of VirE1 is prone to aggregation and forms disordered filamentous structures [8]. This may represent the biologically active form if the protein is secreted independently of the T-strand and without VirE1. In the presence of ssDNA, VirE2 adopts a more ordered solenoidal form with N to C terminal contacts azimuthally around a hollow core [6,7,9]. The structure of the VirE1–VirE2 complex was investigated by X-ray crystallography [20]. The protein contains two major folded domains of similar structure, joined by a flexible linker. The two domains clamp tightly a single alpha helix in VirE1, constraining the termini to an orientation incompatible with oligomerization.

In the plant cell the T-DNA is thought to be directed to the nucleus by VirE2 as well as nuclear localization signals (NLS) on VirD2 [12,21]. T-complex labeling in vitro was described in the literature, producing 'artificial' fluorescent T-complex. In one study, VirE2 was bound to fluorescently labeled ssDNA to produce the T-complex in vitro, which was then microinjected into Tradescantia stamen hair cells. These complexes reached the nucleus [22], while fluorescent ssDNA injected independently of VirE2 remained cytoplasmic. Other evidence indicate a cytoplasmic localization of VirE2, however [14,23], motivating further examination by direct imaging.

In vivo visualization of VirE2 was reported recently. The approach taken was based on split green fluorescent protein, either in a bimolecular fluorescence complementation (BiFC) mode or by opening the beta barrel at sheet 11 [24–26] following Cabantous [27]. VirE2 was found in both cytoplasm and nucleus of Nicotiana benthamiana cells [24,26], but only in the cytoplasm of Saccharomyces cerevisiae [24,25]. However, these studies detected VirE2 not earlier than one or more days post-infection by the bacterium. Transcription of the transgenes occurs with as little as two hours co-inoculation [28], implying delivery of both T-DNA and VirE2 on that time scale.

Here we present an alternative approach to labeling of VirE2 in vitro and in vivo by introducing a tetracysteine (TC) motif to a flexible loop in the protein. The TC motif is recognized by a non-fluorescent biarsenical derivative of Resorufin, known as ReAsH [29]. A similar approach was employed to follow Shigella flexneri effector secretion to human tissue culture cells using the related reagent FlAsH [30]. We show that the VirE2-TC protein displays the expected biochemical interactions and is biologically functional for transformation. We detected it abundantly in live bacteria, and at low abundance in infected plant tissue.
Materials and methods

Bacterial strains and growth conditions

E. coli DH5α and BL21 bacteria were grown in LB medium [31], overnight at 37˚C in a shaker (250 rpm). Agrobacterium strains are listed in Table 1. Agrobacterium were grown in YEP [32] antibiotic (kanamycin 50 μg/ml, carbenicillin 100 μg/ml spectinomycin 250 μg/ml streptomycin 250 μg/ml) overnight at 28˚C, in a shaker (250 rpm). For VirE2-TC expression in the AT12516 (virE2- mutant) strain, bacteria were grown overnight in YEP medium at 28˚C, in a shaker (250 rpm). Cells were then diluted 1:25 in AB minimal medium [33] + 0.5% glucose and grown until OD600 = 0.6, then cells were diluted to OD600 = 0.2 in AB induction medium (AB + 0.5% glucose +200 μM acetosyringone +50 mM MES pH 5.5) overnight at 20˚C in a shaker (250 rpm).

Cloning of wild type virE2 and virE2-TC genes. All PCR reactions were performed using high fidelity DNA polymerase, Pwo (Roche) for small DNA fragments, and Phusion (FINNZYMES) for larger ones, using primers reported in Table 2. For in vitro experiments, wild-type nopaline virE2 was cloned into pHIS Parallell1 plasmid [34] in NcoI and SalI restriction sites with 6X-His tag in E.coli DH5α strain. For virE2-TC cloning, the TC motif was inserted in virE2 gene according to Adams [35], Cys-Cys-Pro-Gly-Cys-Cys, using two steps of PCR.

For in vivo experiments, the virE2-TC gene under the virB promoter was cloned into a binary plasmid pCAMBIA2301 for expression in the bacterium (i.e., not in the T-DNA). The nopaline virB promoter sequence was taken from pDW029 plasmid [36]. The virE2 gene with virB promoter was cloned into the SgrAI restriction site in order to synchronize its expression with the other virulence genes. Wild-type VirE2 was cloned similarly. pCAMBIA2301:VirE2-TC and pCAMBIA2301:VirE2 were transformed into the VirE2 null strain AT12516.

Expression and purification of wild type VirE2 and VirE2-TC proteins

Recombinant proteins were purified from inclusion bodies under denaturing conditions in buffer containing 6M guanidine hydrochloride, in order to prevent aggregation and
precipitation. The wild type virE2 and virE2-TC nopaline genes were cloned in pHis parallel1 plasmid [34] with 6x-His tags between the restriction sites Nco1 and Sal1 and the resulting plasmids were introduced into BL21 (DE3) for protein production. Five ml overnight cultures were inoculated into 500 ml LB and resulting cultures were grown to an OD \(_{600} = 0.6\). Isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM, and resulting cultures were incubated at 37˚C for 3h to induce synthesis of VirE2 or at 30˚C for 5h for VirE2-TC production. Cells were harvested, suspended in buffer A (20 mM sodium phosphate, 500 mM NaCl, pH 7.4) and sonicated. Inclusion bodies were collected by centrifugation, washed twice with buffer A, and denatured by suspension in buffer A + 6M guanidine hydrochloride and 20 mM imidazole for 30 min at room temperature. The denatured proteins were loaded on His-Trap HP 1ml column (GE Healthcare), and soluble proteins were purified using AKTA-basic system (GE Healthcare). The column was washed with 10 column volumes (CV) of buffer A + 20 mM imidazole, and bound His-tagged proteins were eluted from the column using a linear gradient (20 mM to 500 Mm) of imidazole in buffer A. Fractions were collected and refolded by dialysis against 2 L of buffer B (100 mM NaCl, 40 mM sodium phosphate, 10% glycerol, 1 mM DTT, pH 7.4) and analyzed by SDS-PAGE.

VirE1-VirE2-TC co-expression and purification

VirE2-TC was cloned into pACYCDuet-1:VirE1 (obtained from the Israel Structural Proteomics Center, Weizmann Institute of Science). VirE1 was expressed with 6x-His tag while VirE2-TC was untagged. The plasmid was transformed into E.coli BL21 (DE3) strain for protein synthesis. Cells were grown overnight, diluted in the morning 1:100 into 500 ml LB, and regrown until OD\(_{600} = 0.6\). Cells were induced overnight at 16˚C by adding Isopropyl \(\beta\)-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. Cells were harvested and suspended with 20 ml of buffer A and protease inhibitor cocktail (Sigma). The suspended cells were sonicated and centrifuged, the supernatant was loaded on a His-Trap HP 1ml column (GE healthcare) and purified using the AKTA-basic system (GE Healthcare). The column was washed with 10 column volumes (CV) of buffer A + 20 mM imidazole and bound His-tagged proteins were eluted using a linear gradient (20 mM to 500 Mm) of imidazole in buffer A + 6 M guanidine hydrochloride. Fractions were collected and refolded by dialysis against 2 L of buffer B (100 mM NaCl, 40 mM sodium phosphate, 10% glycerol, 1 mM DTT, pH 7.4) and analyzed by SDS-PAGE.

Table 2. List of primers.

| Name          | Sequence                                                                 | uses                                             |
|---------------|--------------------------------------------------------------------------|--------------------------------------------------|
| NCOI F        | 5'–GGC GCC ATG GAT CCG AAG GCC GCC AAT – 3'                              | Cloning of vvirE2 into pHIS vector               |
| SAL R         | 5'–CTC GTC GAC GCT ACA GAC CAG TTA CGG TTA– 3'                          | Cloning of vvirE2 into pHIS vector               |
| VIRE2 mut F  | 5'–TGG TGG CCA GCC TGG TGG CAG TTA CGG GCC GAA CTC TCA– 3'              | Introduction of tetracysteine codons into virE2 |
| VIRE2 mut R  | 5'–ACA GCA GCC TGG ACA GCA CTC GCC GCC GAA CTC TCA– 3'                  | Introduction of tetracysteine codons into virE2virE2 |
| Nde1 F        | 5'–GGC CAT ATG GAT CCG AAG GCC GCC AAT– 3'                              | Cloning of virE2-TC into pACYDuet-1:virE1 vector |
| Kpn1 R        | 5'–GTC GGT ACC CTA CAG ACT GGT TAC GGT TGG– 3'                          | Cloning of virE2-TC into pACYDuet-1:virE1 vector |
| virB F        | 5'–TGG CCC GCG ACA GCG TTA TGT CCA– 3'                                  | Cloning of virB promoter+virE2 or virE2-TC into pCAMBA2301 vector |
| virB R        | 5'–CGA CCC GCG CTA CAG ACT GTT TAC– 3'                                  | Cloning of virB promoter+virE2 or virE2-TC into pCAMBA2301 vector |

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Electrophoretic gel mobility shift assay of protein-ssDNA binding

Purified VirE2 and VirE2-TC proteins were mixed with M13 ssDNA in binding buffer as described below. The mixing ratios (w/w) ssDNA:protein were 1:1, 1:5, 1:10 and 1:15. Samples were incubated overnight at 4˚C for DNA-protein complex formation, and then analyzed by electrophoresis through an 0.8% agarose gel and ethidium bromide staining. The stained gel was scanned in FUJIFILIM FLA-5100 using a green laser (543 nm) and analyzed by Multi Gauge software.

Transmission electron microscopy analysis of VirE2-ssDNA binding

M13mp18 single strand DNA (New England Bio-labs) was denatured by heating at 70˚C for five min and then cooled on ice for five min. Wild-type VirE2 and VirE2-TC proteins were added to the ssDNA in binding buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) at a ratio of 1:10 (w/w) ssDNA:protein for full coverage of the DNA. The ssDNA-protein samples were incubated for 3h or overnight at 4˚C to form the complex. For TEM analysis, 5 μl of the ssDNA-protein complex was placed on glow discharged carbon coated copper grids and negatively stained with 1% uranyl acetate for 50 sec. Samples were imaged in a Tecnai Spirit BioTWIN (FEI) operating at 120 kV. Images were recorded with an Eagle 2K × 2K CCD camera (FEI).

In vitro labeling of VirE2-TC

The VirE2-TC protein was labeled in vitro by ReAsH reagent (obtained from Invitrogen as Lumio Red In-Cell labeling kit, cat no 12589–040). M13 ssDNA was first labeled with Alexa Fluor 488 dye (ULYSIS nucleic acid labeling kit, Molecular Probes) and then mixed with purified proteins, VirE2-TC and VirE2 as a control at room temperature for 3h or overnight at 4˚C. The assembled complexes were labeled with ReAsH reagent at a final concentration of 100 nM. These reactions were incubated for 3 h in darkness to produce fluorescent VirE2-TC. For imaging by laser scanning confocal microscopy (Olympus Fluoview 300 with 60x/1.4NA oil-immersion objective), the complexes were mixed with 3% agarose to yield a final concentration of 1% agarose and hardened quickly into a gel inside the sample chamber. Samples were excited at two wavelengths, 488 nm for Alexa Fluor 488 and 543 nm for ReAsH. Fluorescence emission was recorded at 510–530 nm for Alexa Fluor 488-DNA and at 600–620 nm for the ReAsH-VirE2-TC.

In vivo labeling of VirE2-TC in Agrobacterium

For in vivo labeling of VirE2-TC protein inside the bacteria, the virE2 Agrobacterium strain AT12516 [37] carrying the virE2-TC gene was induced in minimal medium (see growth conditions). The induced cells were washed once with 50 mM HEPES (pH 8) and treated with 650 μM BAL (2,3-dimercaptopropanol) in 50 mM HEPES for 15 min to prevent the nonspecific binding of ReAsH reagent. Then cells were washed with 50 mM HEPES, and 0.5–2 μM of ReAsH reagent was added at the final OD600 0.5. Cells were incubated for 3 h at room temperature in darkness and then washed three times with 250 μM BAL and once with 250 μM BAL plus 20 μM Disperse Blue 3 (obtained from Invitrogen) for removal of excess reagent. Cell samples (5 μl) were placed onto a glass slide and analyzed by laser scanning confocal microscopy using 543 nm excitation and emission detection at 600–620 nm.

In vivo labeling of VirE2-TC in plant cells

Agrobacterium strain AT12516 carrying the virE2-TC gene was induced in LB- MES media (see plant transformation methods). Cells were infiltrated to Nicotiana tabacum and Nicotiana
benthamiana leaves. At 4 to 20 hours post-infection, hand cut tissues were subjected to ReAsH labeling. Tissues were pretreated with 650 μM BAL for 15 min to reduce the nonspecific binding of the reagent, rinsed, and incubated for 30–45 min with 1–2 μM ReAsH in water in darkness. Treated tissues were washed three times with 250 μM BAL and once with 250 μM BAL plus 20 μM Disperse Blue 3. Labeled tissues were analyzed by laser scanning confocal microscopy (Olympus Fluoview 300) using a 60x/1.2NA water-immersion objective. Fluorescence and transmitted light differential interference contrast images were collected simultaneously with a spatial sampling of 90 nm/pixel.

**3D image processing and intensity analysis**

Three dimensional (XYZ) recordings were analyzed offline by the following procedure. Image stacks were first projected (as maximum intensity) down the Z axis in order to find all the fluorescent spots. The slices generating each individual spot were then cropped and inspected visually, together with the differential interference contrast channel that was acquired simultaneously. Fluorescent particles smaller than the diffraction limit of the microscope display the characteristic size and shape of the 3D confocal point spread function. The total spot intensities, on the other hand, reflect the density of fluorophores within the diffraction-limited spot. Therefore projected intensities in the cropped spots (as integrated sum) were tabulated for each detected particle as a measure of the quantity of ReAsH-labeled VirE2. Images were analyzed by Fiji [38] and plots prepared in Origin software (OriginLab Corp).

**Plant transformation methods**

Agroinfiltration assay: *Nicotiana tabacum* and *Nicotiana benthamiana* leaves. Overnight cultures of *Agrobacterium* diluted 1:20 into LB-MES buffer pH 5.9 with 20 μM acetosyringone and resulting cultures were incubated to a final OD$_{600}$ = 0.5–0.6. Cells were pelleted and resuspended in a solution containing: 10 mM MgCl$_2$, 10 mM MES, and 150 μM acetosyringone, incubated at room temperature for 2–3 h and infiltrated with a 1 ml syringe into the leaves.

*Arabidopsis thaliana* root transformation: Transient expression of Beta-glucuronidases-GUS in *Arabidopsis* roots was detected by treatment with GUS substrate 5-bromo-4-chloro-3-indolyl glucuronide (x-gluc) as previously described [39].

Complementation assay: Functionality of VirE2-TC in mediating successful transfer of T-DNA to plants was assessed by extracellular complementation as follows. Two AT12516 (virE2) strains, one carrying a T-DNA binary vector encoding the GFP protein and a second encoding either VirE2 or VirE2-TC, were induced with acetosyringone. The cultures were mixed and infiltrated into *Nicotiana tabacum* plant leaves. Successful transfer of T-DNA encoding GFP by the first bacterial strain to the plant nucleus requires co-transfer of a functional form of VirE2 (or VirE2-TC) by the second bacterial strain into the same plant cell. To confirm co-transfer of T-DNA and functional VirE2 (or VirE2-TC), plant cells were monitored for GFP production monitored after 48 h of infection by laser scanning confocal microscopy.

**Results**

**Molecular interactions of VirE2-TC in vitro**

We introduced tetracysteine (TC) motif Cys-Cys-Pro-Gly-Cys-Cys replacing amino acids 339 to 344 of VirE2 to generate VirE2-TC. This region represents an unstructured linker between the two major folded domains of the protein, as shown by the VirE1-VirE2 crystal structure [20]. We first analyzed biochemical properties of VirE2 and VirE2-TC in vitro by addition of
6x-His tags and purification by immobilized metal affinity chromatography (IMAC). In the absence of its small acidic chaperone VirE1, VirE2 has a strong tendency to oligomerize into poorly soluble, irregular filaments [8]. Therefore purification was performed from inclusion bodies. VirE2 and VirE2-TC co-migrated in SDS-polyacrylamide gels, although VirE2-TC purified at lower concentrations than VirE2 (Fig 1A).

**VirE1-VirE2-TC interaction in co-expression**

We tested for an interaction between VirE2-TC and VirE1 by coproducing VirE2-TC and and 6xHis tagged VirE1 in the same cell following Dym et al [20]. Due to high solubility of the heterodimer protein, purification was performed under native conditions. VirE1 interacted similarly with wild-type VirE2 and VirE2-TC (Fig 1B). The acidic VirE1 runs in gel electrophoresis with a misleadingly large apparent molecular weight. MALDI mass spectrometry analysis confirmed that the lower bands in Fig 1B are indeed VirE1 (data not shown).

**ssDNA–VirE2 and ssDNA–VirE2–TC interaction assays**

Next, we tested for ssDNA binding activity of VirE2-TC by electrophoretic mobility shift assay (EMSA) and transmission electron microscopy (TEM). For EMSA, VirE2 and VirE2-TC were mixed at a series of increasing protein concentrations with M13 circular ssDNA. Both proteins shifted the ssDNA species in agarose gels, although VirE2-TC was less efficient in ssDNA-binding than the native protein (Fig 2). When analyzed by TEM, we detected full coverage of VirE2 bound along the M13 ssDNA substrate (Fig 3A). VirE2-TC, however, bound in shorter segments along the substrate and a considerable amount of protein was detected in the background (Fig 3B). Thus, VirE2-TC binds single-stranded DNA, albeit less efficiently than VirE2, and also forms solenoidal capsids characteristic of the native protein.
Biological function of VirE2-TC in plants

The biological function of VirE2-TC was assayed in *Nicotiana tabacum* leaves and in *Arabidopsis* roots. Two strategies were employed based on binary plasmids introduced into the virE2 mutant strain AT12516. First, we monitored transfer of T-DNA bearing a GUS reporter gene by assaying for GUS activity in infected *Arabidopsis* roots. The infecting bacterium carried the GUS T-DNA binary vector pCAMBIA2301 that was engineered to express either VirE2-TC or native wtVirE2 in the bacterium, from the virB promoter. Roots infected with either strain exhibited similar blue staining indicative of successful delivery of the GUS reporter gene and its expression in the plant cells (Fig 4A and 4B). In control experiments, wild-type A.

Fig 2. Electrophoretic Mobility Shift Analysis (EMSA) confirms binding of VirE2 to M13 ssDNA substrate. M13 ssDNA concentration was held constant, while relative protein concentration increased. Lanes: (A) M13 alone, B-E) wtVirE2 at 1:1, 1:5, 1:10, 1:15 ratios wt:wt, (F-I) VirE2-TC at equal ratios. VirE2-TC binds M13 ssDNA less avidly than the wild-type, but leaves no evidence of completely unbound ssDNA.

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BioAsH detection of VirE2 secretion
tumefaciens carrying pCAMBIA2301 elicited GUS reporter activity in plants upon infection (Fig 4C), whereas *A. tumefaciens* AT12516 carrying pCAMBIA2301 but lacking genes for VirE2 or VirE2-TC failed to elicit GUS reporter activity (Fig 4D).

Next, we monitored transfer of T-DNA bearing a GFP reporter gene to *Nicotiana tabacum* leaves by mixed infection. In this assay, plant tissue is inoculated with a mixture of two strains, one proficient for transfer of T-DNA (encoding GFP for expression in the plant) and the second for transfer of VirE2 or VirE2-TC protein. If the two strains deliver their respective substrates into the same plant cell, complex formation in the plant results in successful delivery of the T-DNA to the plant nucleus and synthesis of the GFP reporter. Fig 5 demonstrates biological function of VirE2-TC in gene transfer. Three bacterial strains were prepared in the *virE2* null background AT12516: one containing a pCAMBIA binary plasmid carrying only the T-DNA(GFP) sequence, one expressing VirE2-TC for secretion, and expressing wtVirE2 for secretion. Additionally, the EHA105 strain (expressing wtVirE2) was transformed with T-DNA(GFP) as a positive control. This positive control (Fig 5A) shows the distribution of expressed GFP delivered in a single infection. Mixed infection by a strain carrying only T-DNA with a second strain expressing either VirE2-TC (Fig 5B) or wtVirE2 (Fig 5C) showed similar GFP expression patterns. In control experiments, infections with the strain carrying T-DNA(GFP) alone failed to incite GFP production (data not shown).

Taken together, results of the *Arabidopsis* root and *N. tabacum* leaf transformation studies establish that *A. tumefaciens* efficiently delivers VirE2-TC to plant cells and, furthermore, that VirE2-TC exhibits WT protein function in mediating T-DNA transfer to the plant nucleus.
**In vitro** labeling of T-complex

Having shown that VirE2-TC interacts with VirE1 and binds ssDNA *in vitro*, and displays biological function in plant transformation assays, we next tested for labeling of the tetracysteine motif with the ReAsH reagent. ReAsH binding to VirE2-TC protein was assayed with complexes formed on M13 ssDNA pre-labeled with Alexa Fluor 488 dye (See Materials and methods). Complexes formed with native VirE2 on fluorescent DNA served as a negative control against non-specific binding of the ReAsH. As shown in Fig 6A, complexes composed of native VirE2 and DNA exhibited fluorescence of the labeled DNA substrate, but no detectable ReAsH fluorescent signal. By contrast, complexes composed of VirE2-TC and DNA, exhibited fluorescent VirE2-TC spots that colocalized with the labeled DNA substrate when analyzed by laser scanning confocal microscopy (Fig 6B).

**In vivo** labeling of VirE2-TC protein in *Agrobacterium*

We assayed for in vivo binding of the ReAsH reagent to VirE2-TC in *A. tumefaciens* as follows. AT12516 carrying pCAMBIA2301 with P<sub>virB</sub>::virE2-TC was treated with acetosyringone to induce synthesis of VirE2-TC. We then treated cells with BAL (2,3-dimercaptopropanol), a reducing agent shown previously to prevent nonspecific cysteine labeling by the ReAsH reagent [40]. Following a wash step, cells were treated with the ReAsH reagent, which is membrane permeable and thus can enter the cell without permeabilization. As shown in Fig 7, approximately 5–15% of the treated cells were fluorescent, whereas similarly treated isogenic cells producing native VirE2 remained dark (data not shown). Thus, the VirE2-TC protein is abundantly labeled with the fluorescent reporter in induced *A. tumefaciens* cells.

**In vivo** labeling of VirE2-TC in plants

Our ultimate aim was to visualize transferred VirE2 in plant cells *in vivo*. To this end, *Nicotiana tabacum* and *Nicotiana benthamiana* leaves were inoculated with AT12516 carrying pCAMBIA2301 with P<sub>virB</sub>::virE2-TC. Within 4 to 16 hours after inoculation, leaf tissues were cut and treated with ReAsH reagent without fixation. Three dimensional data sets were acquired in the confocal microscope. We detected punctate red signals in the cytoplasm after detailed review of the recordings and analysis with the 3D objects counter [41] in Fiji software.
**Fig 6.** Confocal microscopy images of ssDNA-protein particles formed by M13 circular ssDNA labeled with Alexa 488. (A) wild type VirE2, (B) VirE2-TC. Scale bar 50 μm.

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**Fig 7.** *In vivo* labeling of VirE2-TC. ReAsH labeling of virulence activated bacteria expressing VirE2-TC. Inset: zoom of a single fluorescent bacterium (white arrow) shows polar accumulation of the fluorescent label. Scale bar 10 μm.

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This tool provides a number of parameters including the integrated intensity and volume of the selected objects. By comparing brightness, size, and location of the spots as seen in the transmitted light images, we were able to distinguish between VirE2-TC protein within the plant cell and whole bacteria containing VirE2-TC. In particular, the whole bacteria were found on the leaf surface, while fluorescent protein puncta were found within the tissue. No such spots were detected in scans of comparable areas of leaf tissue infected with wild-type VirE2 and similarly treated with ReAsH.

A small gallery of images with intensity analysis appears in Fig 8. Below each fluorescent panel, the same region is shown by differential interference contrast (DIC). Bacteria are strongly refractive and appear dark, whereas the small puncta originate from an object too small to detect by DIC. Fluorescent signals shown were detected in live cell imaging between 4 and 8 hours post inoculation, which is of relevance to rapid transformation kinetics [28]. Full image overlays showing the particles in cellular context appear in Fig 9. Fluorescent VirE2-TC containing particles were observed only in the cytoplasm, whereas the T-DNA must reach the nucleus in order to effect a transformation.

As an additional negative control, we tested a strain of Agrobacterium with the virB operon deleted (PC1000). This strain expresses wild-type VirE2 but is unable to secrete it. We confirmed avirulence by transformation with the binary plasmid (pDGB2_alpha2) for expression of YFP in the host; no YFP fluorescence observed after infiltration to Nicotiana benthamiana leaves. The strain was then transformed similarly to AT12516 with P_{virB}:virE2-TC in pCAM-BIA2301. Fluorescent bacteria were observed in the plant tissue, confirming expression of the VirE2-TC protein (Fig 10), but smaller puncta corresponding to VirE2-TC were not detected in the plant cells.

Discussion

A number of important roles are attributed to VirE2 during the infection process of Agrobacterium tumefaciens [42]. In particular it is believed to function as a capsid by protecting the single strand transfer DNA from degradation in the host cell until it reaches the nucleus. Also notable is that VirE2 synthesized in the plant cell supports efficient transformation by A. tumefaciens virE2- mutants [11], consistent with a protective role in the cytoplasm.

Recent breakthroughs in imaging have enabled the visualization of bacterial secretion in a growing number of cases. For example the conjugative transfer of DNA between E. coli was detected specifically via hemi-methylation [43]. Secretion of disease effectors to eukaryotic cells was visualized elegantly with enzymatic amplification of the signal [44]. Bimolecular fluorescence complementation (BiFC) and derivative approaches based on split barrels of GFP-type fluorescent proteins offer a means to detect transferred polypeptides directly [45–47]. Two groups have recently addressed secretion of VirE2 by these means, which enabled real-time tracking of the secreted protein within the host cells [24–26]. A potential pitfall to the GFP complementation approach is that the maturation time to obtain fluorescence reaches several hours [27], by which time the first steps of transformation may already have been completed [48]. A more general issue is that the host plant must be modified to express the split GFP.

In this work we modified VirE2 with a tetracysteine motif that allows direct in vivo and in vitro labeling using the ReAsH reagent. The related FlAsH reagent was used previously to follow protein secretion by the type 3 secretion system to mammalian cells [30]. One of the advantages of the tetracysteine tags is their small size, as well as the size of the protein-modifying peptide. VirE2 makes N to C terminal “head to tail” contacts in the assembled T-complex, so we chose to insert the tetracysteine peptide within a flexible loop between the major folded
Fig 8. Analysis of ReAsH labeling in leaf tissue to distinguish bacteria from fluorescent VirE2 puncta. (A) Integrated density values of VirE2-TC particles and bacteria labeled with ReAsH reagent. Bars 6 and 8 represent bacteria while the others are VirE2 particles. Bar 7 shows a number of clustered VirE2 particles. Note that the scale is logarithmic in order to display the small particles and bacteria together. (B) Line profiles across a bacterium and several VirE2 particles accentuate the differences in size and total brightness. Note that fluorescent spots of VirE2 are diffraction-limited by the microscope optics. (C) A subset of cropped images showing a bacterium (left) and several VirE2 particles at the same brightness scale. The bacterium image reaches saturation in the figure in order to provide visible contrast in the faint particles. The corresponding region in the differential interference contrast image (acquired simultaneously) is shown below each box. The bacterium appears as a large refractive object while the VirE2 particles are too small to produce a visible signal.

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domains. Because this loop likely serves as a hinge domain for binding alternative substrates, we tested its interactions carefully in vitro. The modified VirE2-TC bound VirE1 comparably to wtVirE2. Binding to ssDNA was partially impaired but not eliminated. Therefore we expected that this tag may not interfere with function or secretion of the protein and, indeed, VirE2-TC was biologically active in replacing native VirE2 in transformation assays.

A disadvantage of the ReAsH reagent is that it labels cysteine-containing proteins to some degree nonspecifically, which has limited its use for plant studies [49]. Background fluorescence can be reduced by treatment of tissues or cells with a reducing agent before and after labeling. We found 2,3-Dimercaptopropanol (BAL) to be much more effective than the standard 1,2-Ethanedithiol (EDT) reagent used for this purpose. Indeed, only treatment with BAL enabled the detection of punctate spots over background fluorescence in ReAsH-treated plant cells. Detection of fluorescent spots in the cytoplasm was highly challenging by standard fluorescence microscopy and was only possible on review of recorded three dimensional stacks. Previous work in HeLa cells appears to have been considerably more straightforward, so that live imaging was feasible [30].

Based on the in vitro data we could estimate roughly what signals might represent VirE2 engaged with a T-strand. Complexes with M13 ssDNA dispersed in a gel typically showed diffraction-limited spots by confocal imaging (Fig 6). This is fully consistent with the size of the circular substrate (7249 bases) in complex with VirE2 [7]. In other words the apparent size of

Fig 9. Overlay views of fluorescent puncta in leaf tissue. (A) infiltrated Agrobacterium labeled with ReAsH, localized near the upper surface of the Nicotiana tabacum leaf. (B-E) VirE2 puncta labeled with ReAsH reagent in Nicotiana tabacum (B,E) and Nicotiana benthamiana (C,D). Brightness settings are adjusted individually for visibility in the overlays. Scale bar 10 μm.

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the spot in the image, approximately 300 nm, reflects the fundamental limitations of microscope optics rather than physical size of the complex. Occasionally the DNA clustered to form somewhat larger spots with notably higher intensity, yet they remained smaller and dimmer than ReAsH labeled bacteria (Fig 8). The Agrobacterium strain used for secretion of VirE2-TC contains two T-DNA segments: the wild-type on the Ti-plasmid and a shorter one present on the pCAMBIA binary plasmid that encodes the GUS protein. These contain approx. 24,000 \cite{50,51} and 5,400 bases respectively, within a small factor comparable to the M13 model substrate. We can estimate the length of the assembled T-complex from previous work showing 80 bases per 5 nm rise \cite{7}, which indicates an upper limit of about 1 μm for the longer T-strand. Given the probability of curling or folding back onto itself, we expect the dimensions of the T-complex to be at or below the resolution limit of the confocal microscope. By comparison with the artificial complexes, the structure-less spots from ReAsH-labeled VirE2 that we

Fig 10. A virB mutant strain does not secrete fluorescent puncta. (A) Large, motile fluorescent spots, visible also in the bright field images (B, lower panels), indicate bacteria. Smaller spots representing secreted VirE2 in the host cells were not observed. Scale bar 10 μm.

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observe in the plants match the expected signal for a T-complex both in apparent size and in fluorescence intensity.

In the complex environment of the leaf tissue, detection of diffraction-limited objects (VirE2 particles) demands slow 3D scanning with a dense pixel rate. (In particular, the image of such an object must cover more than a single pixel in order to distinguish it from noise.) In addition to the fluorescence we acquired a DIC image. Using the combination of signals, bacteria are easily distinguished from molecular objects such as VirE2, which do not appear in the DIC channel. On the other hand, we would be unable to detect motion because of the long time required for the three dimensional scan. It is therefore possible that we missed a fraction of the transferred protein or T-complexes during transit through the plant cytoplasm due to the weak signal that would spread over the field of motion.

In the present study it was possible to compare the VirE2 signal in vivo to that observed in vitro on complexes with defined ssDNA. We consider the major advance to be detection of VirE2 transfer within hours of infection, since transcription of the transgenes initiates on a similar time scale [28]. VirE2 accumulation at longer times might represent excess effector, or priming of the host cells to receive T-DNA from multiple sources. While it has not yet been possible to image the T-strand and VirE2 simultaneously, given the appropriate time scale and the in vitro calibration, the weak, sparse signals detected may represent the secretion relevant to transformation. We expect that future developments in the ReAsH/FlAsH chemistry, in amenable plant model systems, and in the microscopy itself will combine to improve the efficiency of direct substrate detection in the host cell context.

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**References**

1. Chilton MD, Drummond MH, Merio DJ, Sciaky D, Montoya AL, Gordon MP, et al. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell. 1977; 11: 263–271. PMID: 890735

2. Pitzschke A, Hirt H. New insights into an old story: Agrobacterium-induced tumour formation in plants by plant transformation. EMBO J. 2010; 29: 1021–1032. https://doi.org/10.1038/emboj.2010.8 PMID: 20150897
3. Citovsky V, Wong ML, Zambryski P. Cooperative interaction of Agrobacterium VirE2 protein with single-stranded DNA: implications for the T-DNA transfer process. Proc Natl Acad Sci. 1989; 86: 1193–1197. PMID: 2919168

4. Das A. Agrobacterium tumefaciens virE operon encodes a single-stranded DNA-binding protein. Proc Natl Acad Sci U S A. 1988; 85: 2909–2913. PMID: 2452439

5. Gietl C, Koukolíková-Nicola Z, Hohn B. Mobilization of T-DNA from Agrobacterium to plant cells involves a protein that binds single-stranded DNA. Proc Natl Acad Sci U S A. 1987; 84: 9006–9010. PMID: 3480525

6. Citovsky V, Guralnick B, Simon MN, Wall JS. The molecular structure of agrobacterium VirE2-single stranded DNA complexes involved in nuclear import. J Mol Biol. 1997; 271: 718–727. https://doi.org/10.1006/jmbi.1997.1230 PMID: 9299322

7. Abu-Arish A, Frenkiel-Krispin D, Fricke T, Tzfira T, Citovsky V, Wolf SG, et al. Three-dimensional Reconstruction of Agrobacterium VirE2 Protein with Single-stranded DNA. J Biol Chem. 2004; 279: 25359–25363. https://doi.org/10.1074/jbc.M401804200 PMID: 15054095

8. Frenkiel-Krispin D, Wolf SG, Albeck S, Unger T, Jacobovitch J, et al. Plant Transformation by Agrobacterium tumefaciens MODULATION OF SINGLE-STRANDED DNA-VirE2 COMPLEX ASSEMBLY BY VirE1. J Biol Chem. 2007; 282: 3458–3464. https://doi.org/10.1074/jbc.M605270200 PMID: 17060320

9. Bharat TAM, Zbaida D, Eisenstein M, Frankenstejn Z, Mehlman T, Weiner L, et al. Variable Interna l Flexibilit y Character izes the Helical Capsid Formed by Agrobacterium VirE2 Protein on Single-Stranded DNA. Structure. 2013; 21: 1158–1167. https://doi.org/10.1016/j.str.2013.04.027 PMID: 23769668

10. Citovsky V, Zupan J, Warnick D, Zambryski P. Nuclear localization of Agrobacterium VirE2 protein in plant cells. Science. 1992; 256: 1802–1805. PMID: 1615325

11. Citovsky V, Warnick D, Zambryski P. Nuclear import of Agrobacterium VirD2 and VirE2 proteins in maize and tobacco. Proc Natl Acad Sci U S A. 1994; 91: 3210–3214. PMID: 8159726

12. Guralnick B, Thomsen G, Citovsky V. Transport of DNA into the nuclei of Xenopus oocytes by a modified VirE2 protein of Agrobacterium. Plant Cell. 1996; 8: 363–373. https://doi.org/10.1105/tpc.8.3.363 PMID: 8721747

13. Bhattacharjee S, Lee L-Y, Oltmanns H, Cao H, Veena null, Cuperus J, et al. IMPa-4, an Arabidopsis importin alpha isoform, is preferentially involved in agrobacterium-mediated plant transformation. Plant Cell. 2008; 20: 2661–2680. https://doi.org/10.1105/tpc.108.060467 PMID: 18836040

14. Tzfira T, Vaidya M, Citovsky V. VIP1, an Arabidopsis protein that interacts with Agrobacterium VirE2, is involved in VirE2 nuclear import and Agrobacterium infectivity. EMBO J. 2001; 20: 3596–3607. https://doi.org/10.1093/emboj/20.13.3596 PMID: 11432846

15. Maes M, Amit E, Danieli T, Lebendiker M, Loyter A, Friedler A. The disordered region of Arabidopsis VIP1 binds the Agrobacterium VirE2 protein outside its DNA-binding site. Protein Eng Des Sel PEDS. 2014; 27: 439–446. https://doi.org/10.1093/protein/gzu036 PMID: 25221215

16. Otten L, Greve HD, Leemans J, Hain R, Hooykaas P, Schell J. Restoration of virulence of Vir region mutants of Agrobacterium tumefaciens strain B6S3 by coinfection with normal and mutant Agrobacterium strains. Mol Gen Genet MGG. 1984; 195: 159–163.

17. Deng W, Chen L, Peng W-T, Liang X, Sekiguchi S, Gordon MP, et al. VirE1 is a specific molecular chaperone for the exported single-stranded-DNA-binding protein VirE2 in Agrobacterium. Mol Microbiol. 1999; 31: 1795–1807. PMID: 10209751

18. Sundberg C, Meek L, Carroll K, Das A, Ream W. VirE1 protein mediates export of the single-stranded DNA-binding protein VirE2 from Agrobacterium tumefaciens into plant cells. J Bacteriol. 1996; 178: 1207–1212. PMID: 8576060

19. Dym O, Albeck S, Unger T, Jacobovitch J, Branzburg A, Michael Y, et al. Crystal structure of the Agrobacterium virulence complex VirE1-VirE2 reveals a flexible protein that can accommodate different partners. Proc Natl Acad Sci. 2008; 105: 11170–11175. https://doi.org/10.1073/pnas.0801525105 PMID: 18678909

20. Rossi L, Hohn B, Tinland B. The VirD2 protein of Agrobacterium tumefaciens carries nuclear localization signals important for transfer of T-DNA to plant. Mol Gen Genet MGG. 1993; 239: 345–353. PMID: 8391110

21. Zupan JR, Citovsky V, Zambryski P. Agrobacterium VirE2 protein mediates nuclear uptake of single-stranded DNA in plant cells. Proc Natl Acad Sci. 1996; 93: 2392–2397. PMID: 8637884
23. Grange W, Duckely M, Husale S, Jacob S, Engel A, Hegner M. VirE2: a unique ssDNA-compacting molecular machine. PLoS Biol. 2008; 6: e44. https://doi.org/10.1371/journal.pbio.0060044 PMID: 18303950

24. Li X, Yang Q, Tu H, Lim Z, Pan SQ. Direct visualization of Agrobacterium-delivered VirE2 in recipient cells. Plant J Cell Mol Biol. 2014; 77: 487–495.

25. Sakalis PA, van Heusden GPH, Hooykaas PJJ. Visualization of VirE2 protein translocation by the Agrobacterium type IV secretion system into host cells. MicrobiologyOpen. 2014; 3: 104–117. https://doi.org/10.1002/mbo3.152 PMID: 24376037

26. Yang Q, Li X, Tu H, Pan SQ. Agrobacterium-delivered virulence protein VirE2 is trafficked inside host cells via a myosin XI-K-powered ER/actin network. Proc Natl Acad Sci U S A. 2017; 114: 2982–2987; https://doi.org/10.1073/pnas.1610981114 PMID: 28242680

27. Cabantous S, Terwilliger TC, Waldo GS. Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. Nat Biotechnol. 2005; 23: 102–107. https://doi.org/10.1038/nbt1044 PMID: 15580262

28. Narasimhulu SB, Deng XB, Sarria R, Gelvin SB. Early transcription of Agrobacterium T-DNA genes in tobacco and maize. Plant Cell Online. 1996; 8: 873–886.

29. Griffin BA, Adams SR, Tsien RY. Specific covalent labeling of recombinant protein molecules inside live cells. Science. 1998; 281: 269–272. PMID: 9657724

30. Enninga J, Mounier J, Sansonetti P, Nhieu GTV. Secretion of type III effectors into host cells in real time. Nat Methods. 2005; 2: 959–965. https://doi.org/10.1038/nmeth804 PMID: 16299482

31. Bolte S, Cordelières FP. A guided tour into subcellular colocalization analysis in light microscopy. J Microsc. 2006; 224: 213–232. https://doi.org/10.1111/j.1365-2818.2006.01706.x PMID: 17210054

32. Babic A, Lindner AB, Vulic M, Stewart EJ, Radman M. Direct visualization of horizontal gene transfer. Science. 2008; 319: 1533–1536. https://doi.org/10.1126/science.1153498 PMID: 18339441

33. Mills E, Baruch K, Charpentier X, Kobi S, Rosenshine I. Real-time analysis of effector translocation by the type III secretion system of enteropathogenic Escherichia coli. Cell Host Microbe. 2008; 3: 104–113. https://doi.org/10.1016/j.chom.2007.11.007 PMID: 18312845

34. Avilov SV, Moisy D, Naffakh N, Cusack S. Influenza A virus progeny vRNP trafficking in live infected cells studied with the virus-encoded fluorescently tagged PB2 protein. Vaccine. 2012; 30: 7411–7417. https://doi.org/10.1016/j.vaccine.2012.09.077 PMID: 23063830
46. Van Engelenburg SB, Palmer AE. Imaging type-III secretion reveals dynamics and spatial segregation of Salmonella effectors. Nat Methods. 2010; 7: 325–330. https://doi.org/10.1038/nmeth.1437 PMID: 20228815

47. Nickerson A, Huang T, Lin L-J, Nan X. Photoactivated Localization Microscopy with Bimolecular Fluorescence Complementation (BiFC-PALM) for Nanoscale Imaging of Protein-Protein Interactions in Cells. PLOS ONE. 2014; 9: e100589. https://doi.org/10.1371/journal.pone.0100589 PMID: 24963703

48. Gelvin SB. Using BY-2 cells to investigate Agrobacterium-plant interactions. In: Nagata P, Matsuoka D, Inzé P, editors. Tobacco BY-2 cells: from cellular dynamics to omics. Springer Berlin Heidelberg; 2006. pp. 195–206. http://link.springer.com/chapter/10.1007/3-540-32674-X_14

49. Estévez JM, Somerville C. FlAsH-based live-cell fluorescent imaging of synthetic peptides expressed in Arabidopsis and tobacco. BioTechniques. 2006; 41: 569–570, 572–574. PMID: 17140113

50. Barker RF, Idler KB, Thompson DV, Kemp JD. Nucleotide sequence of the T-DNA region from the Agrobacterium tumefaciens octopine Ti plasmid pTi15955. Plant Mol Biol. 1983; 2: 335–350. https://doi.org/10.1007/BF01578595 PMID: 24318453

51. Garfinkel DJ, Simpson RB, Ream LW, White FF, Gordon MP, Nester EW. Genetic analysis of crown gall: fine structure map of the T-DNA by site-directed mutagenesis. Cell. 1981; 27: 143–153. PMID: 6276020

52. Hood EE, Gelvin SB, Melchers LS, Hoekema A. New Agrobacterium helper plasmids for gene transfer to plants. Transgenic Res. 2: 208–218.

53. Fernandez D, Dang TA, Spudich GM, Zhou XR, Berger BR, Christie PJ. The Agrobacterium tumefaciens virB7 gene product, a proposed component of the T-complex transport apparatus, is a membrane-associated lipoprotein exposed at the periplasmic surface. J Bacteriol. 1996; 178: 3156–3167. PMID: 8655494