Agrobacterium infection : translocation of virulence proteins and role of VirF in host cells

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CHAPTER 6

Interaction of the *Agrobacterium tumefaciens* VirF protein with members of the SCF complex

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ABSTRACT

The *Agrobacterium tumefaciens* VirF protein is a prokaryotic F-box containing protein (FBP) that is translocated into host cells via the VirB/VirD4 transport system during the infection process. Interaction with the *Arabidopsis* homologues of the yeast SKP1 protein, ASK1 and ASK2, which was demonstrated using *in vitro* techniques, suggests that VirF plays a role inside the plant cell as part of a SCF complex in degradation of host proteins. Here we performed co-immunoprecipitation experiments to confirm the *in vivo* interaction of VirF to ASK1 in plant cells and to find out whether VirF indeed interacts with the other members of the SCF complex as well. For this purpose we made *A. thaliana* cell cultures expressing *virF,* epitope-tagged *T7-virF*, *HA-virF* and the point mutant *HA-virF*$_{(LP\text{-}AA)}$ with a mutation in the F-box domain. Stable expression of HA-VirF was seen seven weeks after transformation. Whereas the mutant protein was weakly expressed, a high level of HA-VirF was present, which was purified by ion-exchange chromatography and immunoaffinity purification. Immunofluorescence studies suggested that VirF was localized both in the cytoplasm and also in the nucleus. Immunoprecipitation of HA-VirF resulted in co-precipitation of the SCF members ASK1 and CUL1. Our data corroborate that VirF is part of an SCF complex in plant cells and further cements a role for VirF in targeted degradation of (host) proteins during infection.

INTRODUCTION

Concurrent protein synthesis and proteolysis determine the steady state levels of proteins in (eukaryotic) organisms. In eukaryotes protein degradation is mediated by the 26S proteasome. Proteins are often earmarked for degradation by ubiquitination, a post-translational protein modification (Hershko and Ciechanover, 1986; 1998; Willems *et al.,* 2004). In eukaryotes, E3 Ubiquitin ligases are the protein complexes that play an important role in the targeting of substrates for proteolysis by mediating covalent attachment of ubiquitin molecules (poly-ubiquitination) to specific residues of target proteins (Hershko and Ciechanover, 1998). An ubiquitin (Ub) activating enzyme (E1) and a Ub-conjugating enzyme (E2) are responsible for the activation and conjugation of Ub molecules that are finally linked to selected target proteins by an E3-Ub ligase (Figure 1 Chapter 1). To date, two main types of E3-Ub ligases have been described: the HECT (homologous to E6-AP C-terminus) E3, which binds Ub molecules donated by E2 before transferring the Ub moieties to the target protein, and the RING (Really Interesting New Gene) finger E3 enzymes that directly catalyze the Ub transfer from E2-conjugating enzyme to the E3-bound target protein. Among the RING-E3 Ub ligases those forming a so-called SCF complex are particularly prevalent and important. SCF complexes, are made up of a core of three main proteins: Cullin (CUL1), RING-H2 motif containing protein (RBX1), also known as HRT1 or ROC1 (Tyers and Willems, 1999), and the S-phase kinase associated protein/suppressor of kinetochore protein (SKP1), which recruits a fourth subunit called the F-box protein (FBP) (Dharmasiri and Estelle, 2002). The presence of the FBP determines the specificity of the SCF complex, as the FBP recruits the proteins that are targeted for degradation (Patton *et al.,* 1998; Tyers and Willems, 1999).

In *Arabidopsis thaliana* at least 10 Cullins have been reported until now (Bachmair *et al*., 2001). CUL1 or CUL2 form the core of the complex together with RBX1 and one of the 21 described ASK proteins (*Arabidopsis* homologues of the yeast S-phase kinase [SKP1] protein) (reviewed in Moon, Parry and Estelle, 2004; Risseeuw *et al*., 2003). Considering that around 700 genes for FBPs are present in the *Arabidopsis* genome (Andrade *et al.,* 2001; Gagne *et al.,* 2002; Risseeuw *et al*., 2003), protein level regulation seems to be very complex and important in *Arabidopsis*. During reproductive, developmental and defense related processes SCF complexes can play a role autonomously or alternating with other E3-Ubiquitin ligases. In humans, for example, cell cycle progression is highly regulated via SCF^{SKP2} targeting of cell-cycle regulators during the G1-S phase transition. Here, the level of the SKP2 F-box protein is controlled at the M-G1 phase transition through another E3-ligase, the anaphase-promoting complex (APC or cyclosome), coupled to the CDH1 activator protein (Lin and Diehl, 2004). Therefore, protein–protein interactions of FBPs will involve interaction with SKP1 and maybe Cullin subunits from SCF complexes, interaction with substrates targeted for proteolysis and possibly, other E3 ligases.

In plants F-box proteins are connected with several basal functions including regulation of floral development (UFO/FIM) (Leving and Meyerowits, 1995, Ingram *et al*., 1997), circadian rhythms (FKF and ZTL) (Mas *et al*., 2003; Nelson *et al*., 2000; Devoto *et al*., 2002), light signaling (EID and AFR) (Buche *et al*., 2000), self-incompatibility response

[AhSLF-S(2)] (Qiao *et al*., 2004), leaf senescence/lateral branching (ORE9/MAX2) (Woo *et al*., 2001; Ward and Leyser, 2004), as well as in control of auxin (TIR1), gibberelic acid (SLEEPY1), jasmonate (COI1) and ethylene (EBF1/2) signaling (reviewed in Moon, Parry and Estelle, 2004). Some plant pathogens also promote bacterial colonization by using mimicks of FBPs. For instance, *Ralstonia solanacearum* uses a type III secretion system (T3SS) to deliver F-box containing GALA proteins into host cells (Angot *et al.,* 2006). During infection of plants with *Agrobacterium tumefaciens* the bacterial VirF protein is transferred into host cells via the type IV secretion system (T4SS) (Vergunst *et al*., 2000; Schrammeijer *et al*., 2003). VirF carries an N-terminal F-box domain containing the conserved leucine (L26, L38) and proline (P27) residues by which it interacts *in vitro* with ASK1 and ASK2 (Schrammeijer *et al.,* 2001). We hypothesize that VirF is a member of an SCF complex inside host cells that targets proteins for degradation to promote tumorigenesis and *Agrobacterium* infection on the host plant.

Expression of epitope-tagged proteins in plant cell cultures has been widely used to test protein-protein interactions in *in vivo* conditions (Ferrando *et al.,* 2000; Farras *et al*, 2001, Devoto *et al*., 2002). To address whether VirF has a functional role as part of an SCF complex in plant cells, we performed co-immunoprecipitation experiments using *A. thaliana* cell suspension cultures that expressed HA-VirF. Here we demonstrate interaction with two core proteins of the SCF complex, ASK1 and CUL1, showing evidence that *in vivo* a SCF^{VirF} complex is assembled, which is most likely involved in the targeting of protein substrates for degradation during the *Agrobacterium* infection process.

EXPERIMENTAL PROCEDURES

Construction of epitope-tagged VirF

During the course of the study we made four different plasmids to obtain plant cells expressing different forms of VirF (Table 1). We used pRAL7014 (pBDH5::*p35S::virF*) (Regensburg-Tuïnk and Hooykaas, 1993) as source for the wild type *virF* gene. A derivative of this vector containing a T7 tagged version of *virF*, was constructed by introducing upstream of the *virF* gene a *EcoR*V-*Nsi*I linker (5'-ATCGCCATGGCTAGCATGACTG GTGGACAGCAAATGGGTCGCGGATCCAGGCCTAGAAATTCGAGTTTGCGTGATGCA-3'), containing the sequence of the amino terminal end of the T7 phage major capsid protein (Novagen, Madison, USA). To this end, first the *EcoR*V-*Nsi*I linker was cloned in pUC19*::virF,* from which a segment containing the *T7::virF* gene was subcloned as *BspH*I fragment into the *Nco*I site of pMTL24 (Chambers *et al*., 1988). Finally, a *Sal*I fragment from pMTL24::*T7::virF* was inserted in the binary vector pBDH5*,* giving origin to pBDH5::*P35S::T7tag::virF.*

Additional epitope-tagged constructs were made by translational fusion between a human influenza virus hemagglutinine (HA) epitope and the full-length coding sequence (cds) of the *virF* and of the $virF_{(LP\text{-}AA)}$ mutant genes (Schrammeijer *et al*., 2001), resulting in *HA-virF* and $HA\text{-}virF_{(LP\text{-}AA)}$ fusion genes under the control of the *BigMac* promoter (*pBigMac*) (Comai, Moran and Maslyar, 1990; Figure 1). First, a *Bam*HI-*Nsi*I linker (5'-GAT**CCG**AAATT CGAGTTTGCGTGATGCA-3') in which the endogenous ATG start codon of the *virF* gene

was mutated to CCG (*Bam*HI underlined), was cloned in pRAL7088 (Schrammeijer, Hemelaar and Hooykaas, 1998), resulting in pSDM3192. The 3' 150 nucleotides of *virF* were amplified by PCR using primers VirF17 (5'-CCGctcgagGTTATGGCA GAAG-3') and VirF18 (5'-CgagctcTCTCATAGACCGCGCGTTGATCG-3') containing *Xho*I and *Sac*I sites respectively (underlined). Subsequently, the original *virF* flanking region downstream of the *virF* stop codon in pSDM3192, was replaced by the *Xho*I-*Sac*I digested PCR product.

Table 1. Bacterial strains and plasmids used in this study

	Description	Reference
Strains:		
Agrobacterium tumefaciens LBA1100	pAL1100 ΔT_L,ΔT_R , Δ*tra*, Δ*occ.* Rif^{r}Spc	Beijersbergen *et al*., 1992
Agrobacterium tumefaciens GV3101(pMP90RK)	C58C1 chromosomal background carrying pMP90RK helper Ti, a nopaline disarmed derivative of pTiC58. Rif	Koncz and Schell, 1986; Koncz *et al*., 1994
Plasmids:		
pBDH5::T7-*virF*	pBDH5*::p35S::T7 tag::virF*	T. Regensburg-Tuïnk, *unpublished*
pBI426 (pSDM1505)	*gus-nptII* fusion under control of 35S promoter(2X) and with nopaline synthase terminator (T-nos)	William Crosby, NRC Saskatoon, Canada; Datla *et al.*, 1991
pBI770::*virFΔATG*$_{(LP\text{-}AA)}$	GAL4-DNA binding domain fusion with VirF containing two point mutations (Leu and Pro for two Ala residues)	Schrammeijer *et al*., 2001
pCAMBIA2300	*A. tumefaciens* binary vector	Centrum for the Application of Molecular Biology to International Agriculture-CAMBIA, Canberra, Australia
pCGN7344	Binary vector derivative of pCGN7329	Comai L., Moran P. and Maslyar D. 1990
pRAL7088	pUC19::*virF*	Schrammeijer *et al*., 1998
pSDM1542 (pER19)	pCGN7344 *pBigMac-gus* cassette in pIC20H	E. van der Graaf, E. *Unpublished*
pSDM3080	pIC20H::*pBigMac::gus::T-mas*	E. van der Graaf, *unpublished*
pSDM3192	pUC19::*virFΔATG*	B. Schrammeijer, *unpublished*
pSDM3511	pUC19::*pBigMac::HA tag::virFΔATG ::T-nos*	This study
pSDM3541	pCAMBIA2300::*pBigMac::HA tag::virFΔATG ::T-nos*	This study
pSDM3542	pUC19::*pBigMac::HA::virF*$_{(LP\text{-}AA)}$*::T-nos*	This study
pSDM3549	pCAMBIA2300::*pBigMac::HA::virF*$_{(LP\text{-}AA)}$ *::T-nos*	This study

Rif: Rifampicine resistance
Spc: Spectinomycin resístanse

The terminator sequence of the *nopaline synthase* gene (*T-nos*) from pBI426 (William Crosby, NRC Saskatoon, Canada; Datla *et al*., 1991) was cloned as *Sac*I-*EcoR*I downstream of the *virF* sequence. Two complementary HA epitope oligonucleotides corresponding to two peptide chains for the HA epitope linked by a GGA codon (5'-ctagaAAATGGAATATCC

ATATGATGTTCCAGATTATGCTGGATATCCATATGATGTTCCAGATTATGCTAg-3') were annealed, containing sticky ends (underlined) for *XbaI* and *BamH*I. This linker was cloned as *Xba*I-*BamH*I upstream and *in frame* with *virF*. Subsequently, the *pBigMac* promoter region from pSDM3080 was cloned as *Xba*II fragment upstream of the *HA* linker giving origin to pSDM3511. Finally, the complete cassette *pBicMac::HA::VirF::T-nos* from pSDM3511 was cloned as *Xba*I-*EcoR*I (partial) in pCambia2300 (CAMBIA, Australia) resulting in the binary vector pSDM3541.

Next, sequencing of pSDM3511 (Base Clear Laboratories, The Netherlands), revealed a duplication in the *BamH*I site of the *HA* linker. To clone the $VirF_{(LP\text{-}AA)}$ mutant as a HA fusion we followed a similar strategy as for HA-VirF (Figure 1). First, we replaced the 5' 497 nucleotides of the wild type *virF* of the first pSDM3511 cloning step with the corresponding *BamH*I-*Xho*I fragment from pBI770::$virF\Delta ATG_{(LP\ to\ AA)}$ (Schrammeijer *et al*., 2001). In this sequence, the codons encoding the conserved leucine and proline (LP) residues in the F-box are replaced by the alanine (A) encoding codon. After cloning the *Xba*I-*BamH*I *HA* linker upstream of the $VirF_{(LP\text{-}AA)}$ gene, the *EcoR*V-*EcoR*I fragment containing the $HA::VirF_{(LP\text{-}AA)}::T\text{-}nos$ was subcloned in pSDM3511, resulting in pSDM3542. The complete cassette under the control of the *pBicMac* in pSDM3542 was finally cloned in pCambia2300 as *Xba*I-*EcoR*I (partial) giving origin to the binary vector pSDM3549.

To verify the *in frame* fusion of the *HA* linker to *virF* and $VirF_{(LP\text{-}AA)}$, the vectors carrying the genes, pSDM3511 and pSDM3542 respectively, were sequenced (Base Clear Laboratories, The Netherlands). Expression of the fusion proteins in *A. tumefaciens* LBA1100 was tested by Western blot using horseradish peroxidase (HRP) conjugated HA antibodies (anti-HA-HRP conjugate, 1:1000; Roche Diagnostics Nederland B.V.)

Transformation of *Arabidopsis* cell suspensions and plants with *Agrobacterium tumefaciens*

Culture media and conditions for *Agrobacterium* mediated transformation of *A. thaliana* cell suspensions were based on the protocol described by Ferrando *et al.* (2000). *A. tumefaciens* strains LBA1100 (Beijersbergen *et al*., 1992) and GV3101(pMP90RK) (Koncz and Schell, 1986; Koncz *et al.,* 1994) were transformed with pBDH5::*T7-virF*, pRAL7014 (pBDH5::*virF*; Regensburg- Tuïnk and Hooykaas, 1993), pSDM3541 or pSDM3549. Twenty ml of overnight bacterial cultures in YEB medium (Kconcz *et al.,* 1994) containing 250 μg/ml spectinomycin and 100 μg/ml kanamycin (LBA1100), or 100 μg/ml rifampicine and 100 μg/ml kanamycin (GV3101) (28°C, OD_{600} ~1.5), were centrifuged for 15 min at 3000 rpm. The pellet was washed in 20 ml of cell culture medium (CM: 4.4 g MSMO Sigma M6899, 30 g sacharose, pH 5.7) plus 0.5 mg/l of1-naphthalene acetic acid (NAA) and 0.1 mg/l of kinetine (KIN) and finally resuspended in 2 ml (0.1 vol) of CM. Aliquots of 10 ml of *A. thaliana* cell suspensions of wild type and transgenic cultures for *ASK1-myc, CUL1-HA, CUL2-HA, CUL2-HA/ASK1-myc* (Table 2), were collected after one week of subculture, centrifuged (5 min, 1000 rpm) and washed with 30 ml of CM containing NAA (0.5 mg/l) and KIN (0.1 mg/l). Finally, the pellet was resuspended in 50 ml of this medium and for each transformation, mixed with 0.4 ml of *Agrobacterium* culture carrying the *virF, T7virF, HA-virF or HA-*$virF_{(LP\text{-}AA)}$

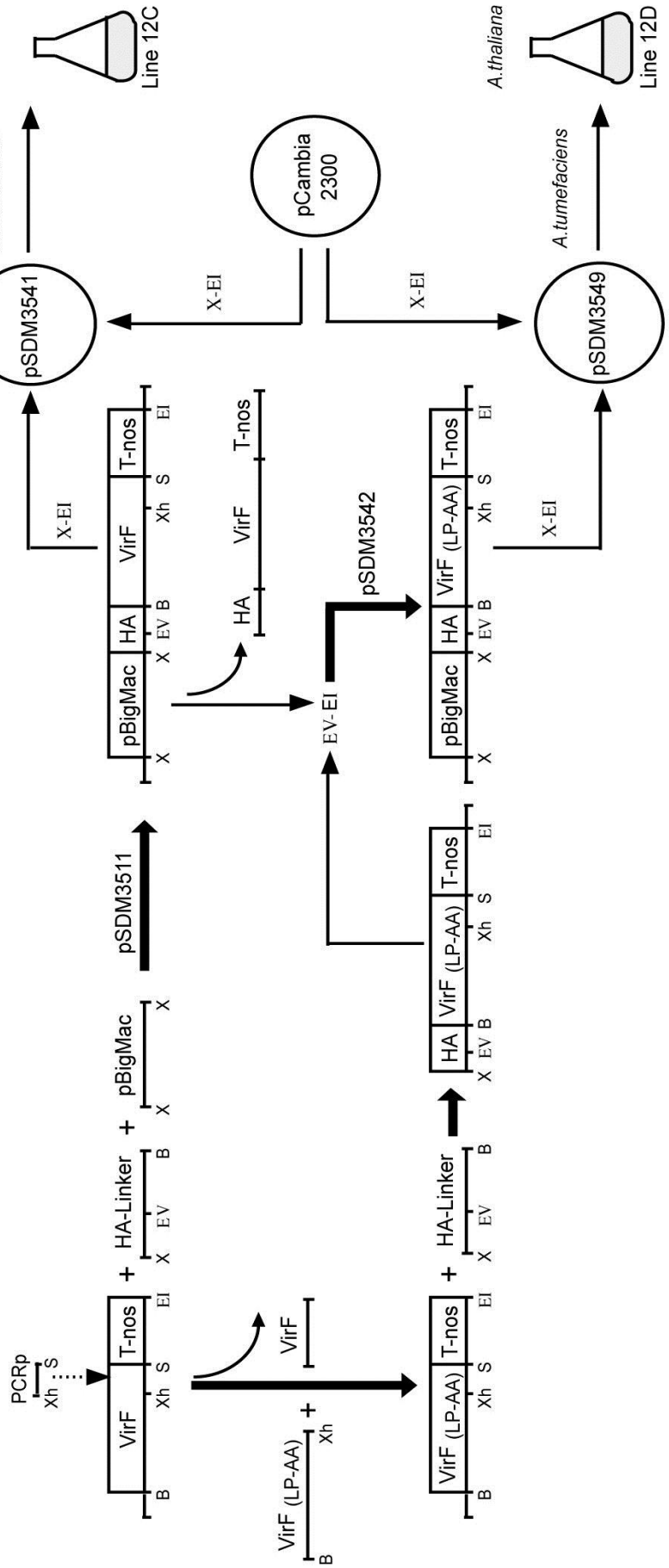

Figure 1. **Schematic representation of the construction of *Arabidopsis thaliana* cell cultures expressing epitope-tagged HA-VirF and HA-VirF$_{(LP-AA)}$** . The *virF* and the *virF*$_{(LP-AA)}$ genes were cloned in pUC19 as translational fusions with HA epitope (pSDM3511 and pSDM3542 respectively) under the control of the pBigMac promoter and the terminator of the nopaline synthase gene (T-nos). Both fusion genes were subcloned in pCambia2300. *A. thaliana* wild type cell suspensions were transformed with *A. tumefaciens* GV3101(pMP90RK) containing the binary vectors pSDM3541 or pSDM3549. Stable cell lines expressing HA-VirF (line 12C) and HA-VirF$_{(LP-AA)}$ (line 12D) were selected. PCRp: product of amplification of the last 150 nucleotides of the *virF* gene, B: *BamHI*, EI: *EcoRI*, EV:*EcoRV*, S: *Sac*I, X: *Xba*I, Xh: *Xho*I.

plasmid. Cells were incubated during two days with a photoperiod of 16 hours (~21°C, 120 rpm). Then, the medium was replaced by fresh CM (NAA/KIN) containing 200 μg/ml of Claforan (Cefotaxime) and 150 μg/ml of Ticarcilline/Clavunilic Acid (mix 15:1). Selection for kanamycin resistance was performed at the fifth day of transformation (50 μg/ml) and continued during the subsequent weekly subcultures. For nomenclature of resulting transformation lines see Table 2.

Table 2. Nomenclature of lines transformed by *A. tumefaciens* GV3101 (pMP90RK)

	Constructs[a]	**A**	**B**	**C**	**D**
Plant Cell Lines[a]		T7-VirF (Km^r)	VirF (Km^r)	HA-VirF (Km^r)	HA-$VirF_{(LP-AA)}$ (Km^r)
7	ASK1-myc (Hyg^r)	7A	7B	7C	7D
8	CUL1-HA (Hyg^r)	8A	-	-	-
9	CUL2-HA (Hyg^r)	9A	-	-	-
10	CUL2-HA/ASK1-myc (Km^r -Hyg^r)	10A	-	-	-
11	CUL2-HA/ASK2-myc (Km^r -Hyg^r)	11A	-	-	-
12	Wild Type	12A	12B	12C	12D

[a] Names of lines transformed by LBA 1100 were similarly designated from 1 (A, B, C, D), 2A, 3A, 4A, 5A till 6 (A, B, C, D).
[r] Kanamycin (Km) or Hygromycin (Hyg) resistance
- No transformed

To perform *in planta* immunocytolocalization analysis, *A. thaliana* plants were also transformed using *A. tumefaciens* strain LBA1100 carrying pSDM3541 (*HA-virF*) or pSDM3549 (*HA-virF$_{(LP-AA)}$*). Transformation was performed according to the floral dip method described by Clough and Bent (1998). Selection of transformants was done in 0.5X MS Medium (0.5X Murashige and Skoog salts, 36.7 mg/l FeNaEDTA, 1X Murashige and Skoog vitamins, 2% sucrose, 100 mg/l inositol, 0.5 g MES, 0.7% Daishin agar (Duchefa,), pH 5.8 containing 70 μg/ml kanamycin and 100 μg/ml timentin.

VirF expression levels in transformed lines were detected on total cell extract analyzed by immunoblot detection using monoclonal anti-HA-HRP conjugate (Roche Diagnostics Nederland B.V; 1:1000). To this end, cell culture samples were collected by filtration and immediately frozen in liquid nitrogen. A tiny amount of cells per sample (approximately 50 mg) was placed in a 1.5 ml microcentrifuge tube (Eppendorf) containing two metal beads and pulverized by vortexing. The extract was resuspended in 100 μl of 1X Loading SDS-PAGE Laemmli buffer (Laemmli, 1970) and boiled for five minutes (100°C). A volume of 10 μl per sample was used for Western blot analysis.

Immunofluorescence microscopy

A. thaliana cells and plant tissue were fixed in 4% formaldehyde, embedded, sectioned and further processed as described by Ferrando *et al*., (2000). Sections were labelled using rat anti-HA, High Affinity (Clone 3F10, Roche Diagnostics Nederland B.V; 1:1000), as primary antibody. The sections were washed with phosphate buffered saline-

0.1% Tween-20 (PBS-T) and treated for 1 hour at 20°C with anti-rat IgG Alexa 488-molecular probe A11006 to visualize HA-VirF or HA-CUL1. Besides the respective antisera, sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for nuclei visualization or alfa-tubulin (Sigma C3181) primary and anti-mouse Cy3 conjugated for microtubules/microfilament detection. Fluorescence analysis was performed using Leica Aristoplan and DMRB microscopes with FICT and DAPI filters, and recorded with a Hitachi HV-20 camera.

Isolation of HA epitope-tagged proteins

Five grams of cell suspensions of lines 12C and 12D (Table 2) was pulverized in liquid Nitrogen and resuspended in 10 ml of ice-cold plant extraction buffer-20 (PEX20: 20% glycerol, 50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.8 mM PMSF and 10 μl/ml of protease inhibitor cocktail [PIC, Sigma]) containing 2 mM of ATP. The suspension was filtered through several layers of Miracloth (Calbiochem) and Nylon mesh (50 and 20 μm of pore size) and centrifuged at 13500 rpm (Sorval SS43 rotor) during 10 minutes. We determined the total protein content for the clarified supernatant by the Bradford protein assay (Bio-Rad laboratories Gmbh-Germany). Protein fractions were then submitted to Ion Exchange Chromatography (IEC) using a DEAE-FF Sepharose precast 1 ml column (Amersham Pharmacia) pre-equilibrated with 20 ml of PEX20 buffer. Washing of the matrix was done with approximately 15 column volumes of wash buffer (WB: 10% glycerol, 50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 2 mM of ATP and 1mM DTT). A five-step gradient solution of WB plus 50 mM, 100 mM, 200 mM, 300 mM and 400 mM NaCl was used for protein elution of the IEC matrix using 2.5X column volume per step. Flow through, washings and NaCl gradient eluates were analyzed using Western blot with monoclonal HA antibodies (Roche Diagnostics Nederland B.V; 1:1000) and secondary conjugated anti-rat IgG (Alexa Fluor Dies series, Invitrogen-Molecular Probes, The Netherlands, 1:1000). Reading was performed in an Odyssey infrared fluorescence scanner (LI-COR Biosciences). HA-positive fractions were combined and purification of HA-VirF was performed using anti-HA affinity matrix (Roche Diagnostics Nederland B.V). To this end, 0.25X volumes of both 100 mM and 200 mM NaCl IEC fractions were mixed and pre-cleared during 1 h using 50 μl of Protein G Sepharose (Sigma-Aldrich) pre-equilibrated three times with 500 μl of WB-150 mM NaCl. Subsequently, the sample was recovered (1000 g/2 min) and placed in contact during 4 h with 50 μl of similarly pre-equilibrated anti-HA matrix under constant rocking. The anti-HA matrix was washed with 100X volumes of WB-150 mM NaCl. Protein elution was done in two steps using 100 μl of elution buffer 500 (E_{500}: 10% glycerol, 50 mM Tris HCL pH 7.5, 10 mM $MgCl_2$, 500 mM NaCl, 1 mM DTT, 10 mM NaF, 0.1 mM Na_3VO_4) followed by 100 μl of a mixture 9:1 of 100 mM glycine pH 2.5 with 1 mM Tris-H-Cl pH 8.0. Presence of the purified protein in the eluted fractions was detected as described above.

Immunoprecipitation analysis

Seventy μl of Affigel-10 agarose matrix (BioRad Laboratories) was washed 3 times with 500 μl of ice-cold Milli-Q water and centrifuged at 1000 g for 3 minutes. 5.6 μg of anti-HA

IgG (Clone 3F10, Roche Diagnostics Nederland B.V) was immobilized to the affigel matrix in 350 μl of coupling buffer (50 mM HEPES pH 7.5, 50 mM NaCl) at 4°C rocking during 4 hours. The coupling reaction was stopped by an additional incubation for 1 h with 10.5 μl of 1 M ethanolamine HCl pH 8. Subsequently, the matrix was washed (1000 g, 3 min) twice with 350 μl of coupling buffer, twice with 500 μl of E_{500} buffer and twice with 500 μl of starting buffer (10% glycerol, 50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 10 mM NaF, 0.1 mM Na_3VO_4). A number of three similarly processed anti-HA matrix aliquots was obtained and stored at 4°C until further use.

After one week of subculture, 200 ml of *A. thaliana* wild type and transformed cell suspensions lines 12C and 12D (Table2), were collected by vacuum filtration and pestle-pulverized in liquid nitrogen. Eight aliquots of approximately 0.25 g per line were further treated with metal beads (0.3 min, 30 1/s frequency) using a RETSCH MM301 grinder apparatus, resuspended in 490 μl ice-cold plant extraction buffer-20 (PEX20: 20% glycerol, 50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 0.8 mM PMSF and 10 μl/ml of protease inhibitor cocktail [PIC, Sigma]) containing 10 mM NaF and 0.1 mM Na_3VO_4 as phosphatase inhibitors and mixed by vortexing. After ice-incubation (10 min), the samples were centrifuged at 20000 g for 20 min and the supernatant clarified by additional centrifugation of 10 min. Four ml of the collected supernatant was placed on pre-washed Vivaspin 6 Polyethersulfone 10000 MWCO tubes (Sartorius Filtratie B.V., Netherlands) and centrifuged during 80 min at 4000 rpm (Jouan CR412 centrifuge). After determination of protein concentration in the three samples, the volume containing 3 mg of total protein was mixed 1:1 with 2X starting buffer containing 0.8 mM PMSF and 10 μl/ml of PIC and placed in contact with anti-HA matrix pre-equilibrated with 500 μl of starting buffer-PMSF-PIC (1000 g/ 3 min). The immunoprecipitation reaction was performed rocking during 4 h at 4°C. The matrix of each reaction was washed using 100 volumes of 1X starting buffer containing 0.08 mM PMSF and 1 μl/ml PIC through micro Bio-Spin chromatography columns (Bio-Rad Laboratories) and finally recovered in micro-centrifuge tubes for subsequent pre-elution with 350 μl of E_{150} buffer (10% glycerol, 50 mM Tris HCl pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT, 10 mM NaF, 0.1 mM Na_3VO_4) and 350 μl of E_{500} containing 0.8 mM PMSF and 10 μl/ml PIC. After the last step, half of the matrix was eluted 3X with 40 μl of 8.0 M Urea. The concentrated extract, the non-eluted matrix, the 8.0 M Urea eluate (U) and the matrix after elution (M) were separated by SDS–PAGE and analyzed by immunoblotting with monoclonal anti-HA-HRP conjugate (Roche Diagnostics Nederland B.V; 1:1000), rabbit polyclonal anti-ASK1 (Ferrando *et al.,* 2000; 1:5000) and rabbit polyclonal anti-CUL1 (Gray *et al*., 1999; 1:15000) for the HA-VirF (12C), the HA-$VirF_{(LP\text{-}AA)}$ (12D) and the wild type *A. thaliana* lines. Anti-rabbit IgG-HRP as secondary antibody conjugate was used in combination with anti-ASK1 and anti-CUL1. To test if additional subunits co-precipitated with HA-VirF, a similar analysis was performed using anti-sera against subunit five of the COP9 signalosome (CSN5), the plant RUB conjugation enzyme (Nedd8), the 20S proteosomal subunit and the tobacco protein kinase (NPK5), plant homologue of the sucrose non-fermenting (Snf1/AMPK) related kinase (SnRK) (Farras *et al*., 2002). Immunoblot detection with HRP conjugated rabbit antiserum was used as negative control. Detection was performed by

chemiluminiscence using HRP substrate (Lumiglu reagent-Cell Signaling Technology, Westburg, The Netherlands).

RESULTS

Strategy, transformation and *in planta* expression of VirF

The presence of an F-box domain in the *A. tumefaciens* VirF protein and its interaction in yeast two-hybrid assays with the *Arabidopsis* homologues of the yeast SKP1 protein, ASK1 and ASK2, suggested that VirF functions as part of a SCF complex inside the plant cell during *Agrobacterium* infection (Schrammeijer *et al.,* 2001). In addition, earlier studies showed that VirF does not interact with ASK1 and ASK2 when the N terminal F-box domain is deleted or a point mutation in the conserved leucine (Leu) and proline (Pro) residues is present in VirF. To confirm the interaction of VirF to ASK1 and determine additional binding to other members of the SCF complex *in planta*, we performed co-immunoprecipitation experiments using plant cell cultures expressing VirF. To this end *A. tumefaciens* strains LBA1100 (Beijersbergen *et al.,* 1992) and GV3101(pMP90RK) (Koncz and Shell, 1986) carrying either plasmid pRAL7014 (pBDH5::*virF,* Regensburg-Tuïnk and Hooykaas, 1993), pBDH5::*T7-virF*, coding for VirF with a T7-tag epitope at the N-terminus, pSDM3541, encoding an HA-tagged VirF, or pSDM3549, corresponding to the HA-tagged $VirF_{(LP-AA)}$ mutant protein, were used to transform the *A. thaliana* cell cultures as described by Ferrando *et al.,* 2000 (Figure 1, Table 2). Besides wild type cultures also lines expressing tagged versions of ASK1 (myc) and CUL1 or CUL2 (HA) were used as recipients.

The nomenclature for the lines transformed with the different constructs is depicted in Table 2. Though 11 out of 24 initially transformed lines showed the 372 bp PCR fragment characteristic for the presence of virF (lines 1C, 1D, 2A, 6D, 7A, 7C, 8A, 12A, 12B, 12C and 12D, data not shown), the expression of the epitope-tagged proteins by Western blot was only visualized in four lines. HA-VirF (~27kDa) was expressed abundantly seven weeks after transformation in lines 7C and 12C, (Figure 2), but no signal was obtained for the lines transformed with *virF* or *T7-virF* (data not shown). A low level of protein HA-$VirF_{(LP-AA)}$ was observed at 7 weeks and at 14 weeks after transformation in lines 7D and 12D, (Figure 2). This signal was not seen at 11 weeks, maybe due to shorter exposure time of the film to the chemiluminiscence reagent. The protein levels remained constantly low during the course of the study, suggesting that the Leu and Pro residues are relevant for stability of VirF in the plant cell. However, the myc-tagged ASK1 could not be detected by immunoblot with myc antibodies in lines 7C and 7D as it had been previous to transformation (data not shown), suggesting instability of ASK1-myc expression after introduction of the *HA-virF* gene. Therefore, only the wild type single transformed lines 12C and 12D expressing HA-VirF and HA-$VirF_{(LP-AA)}$, were subsequently used for further analysis.

Cellular localization of VirF

In cells transiently expressing VirF, VirF was found to display nuclear localization together with VIP1, one of its proposed target host proteins (Tzfira *et al*., 2004), although,

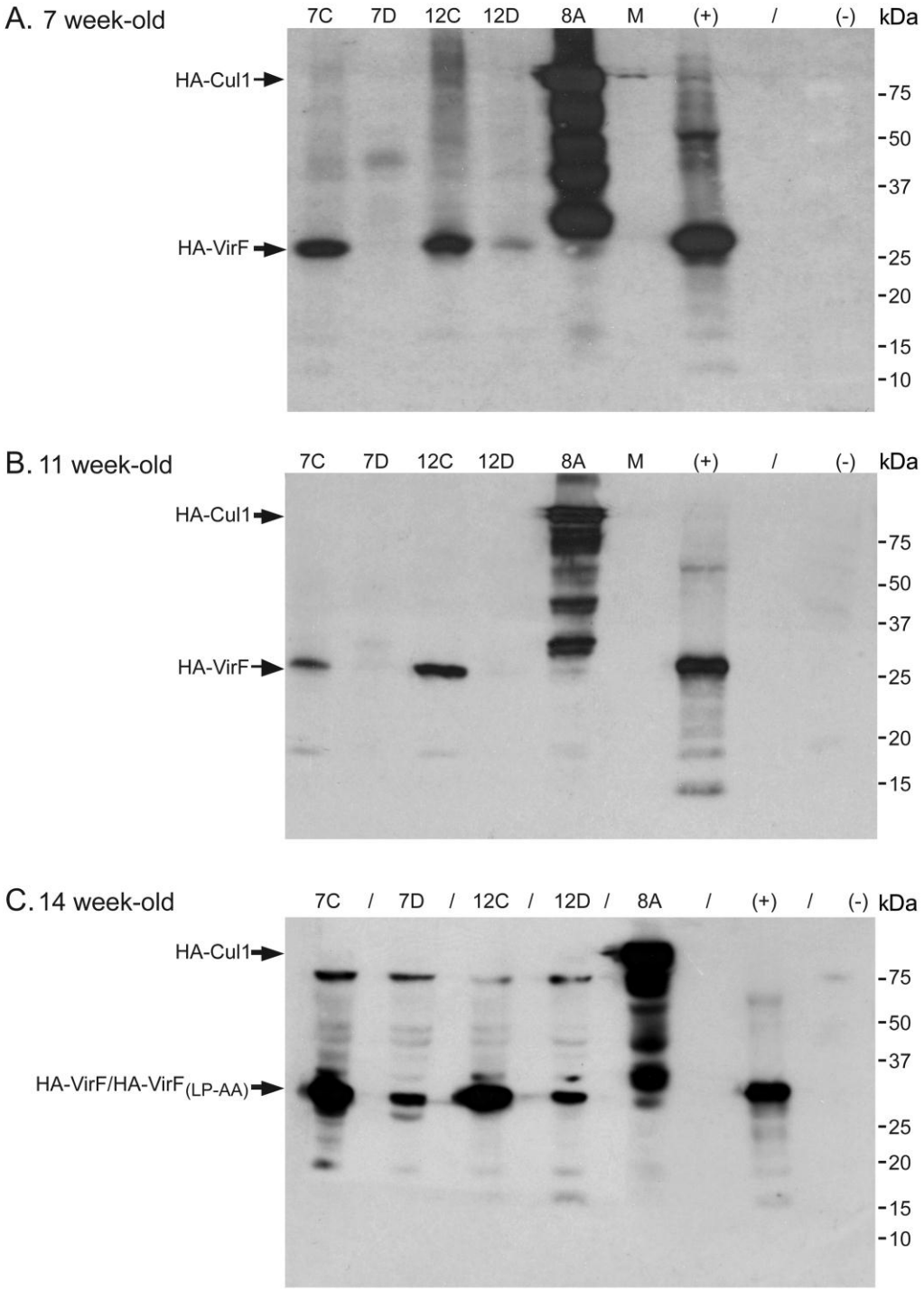

Figure 2. Protein expression of HA epitope-tagged proteins extracted from VirF cell lines. Western blot analysis using HA antibodies was performed on cell cultures transformed with *A. tumefaciens* containing pSDM3541 (*HA-virF*: lines 7C and 12C) and pSDM3549 (*HA:virF*$_{(LP\text{-}AA)}$: lines 7D and 12D) (cell extracts in 1X Laemmli loading buffer). Expression of HA-VirF (~27kDa) was first observed seven weeks after transformation in lines 7C and 12C **(A)** and remained after 11 **(B)** and 14 **(C)** weeks post transformation. The overexposed films detected weak expression of HA-VirF$_{(LP\text{-}AA)}$ at 7 and 11 weeks (data not shown), but this protein was only clearly detected after 14 weeks in lines 7D and 12D **(C).** HA-Cul1 (86 kDa) expressing line 8A and *A. thaliana* HA-VirF expressing plants (+) were used as positive control for HA detection. Wild type *A. thaliana* cells were used as negative control (-). M: protein standard BioRad marker.

VirF does not contain any typical nuclear localization signal (NLS). Earlier we identified several other putative targets of VirF (PIF) (Chapter 3) that are probably present not only in the cytoplasm but also in a variety of other subcellular localizations. Therefore, we analyzed here where VirF may be located using the cell cultures stably expressing HA-VirF.

We compared the fluorescence patterns of wild type *Arabidopsis* cell suspensions with cultures expressing HA-VirF (line 12C). Since the cell cultures expressing CUL1 used HA as epitope tag, we used the line 8A (HA-CUL1, G. Molnar, *unpublished*) as reference pattern for HA-VirF localization. Wild type and transformed *A. thaliana* cultures 12C and 8A were processed for immunocytolocalization as described by Ferrando *et al*, (2000). We observed that in HA-VirF and HA-CUL1 expressing cells, in addition to the immunofluorescence staining pattern of the stained nuclei (DAPI), the signal was also seen in the cytoplasmic region, whereas in the wild type cells signal was not detected neither in the nucleus nor in the cytoplasm (Figure 3). Staining analysis of *A. thaliana* plants expressing HA-VirF confirmed the location of VirF in whole cells of cotyledon vascular tissue (Figure 4a). On the other hand, the immunofluorescence staining patterns of antibodies detecting SCF subunits ASK1 and CUL1 and proteasomal α-subunits (Farras *et al*., 2001), are similar to those observed for VirF, which fluorescence pattern corresponds to the one visualized by anti-tubulin antisera, showing co-localization of VirF with mitotic spindle and phragmoplast during cell division (Figure 4b, c, d).

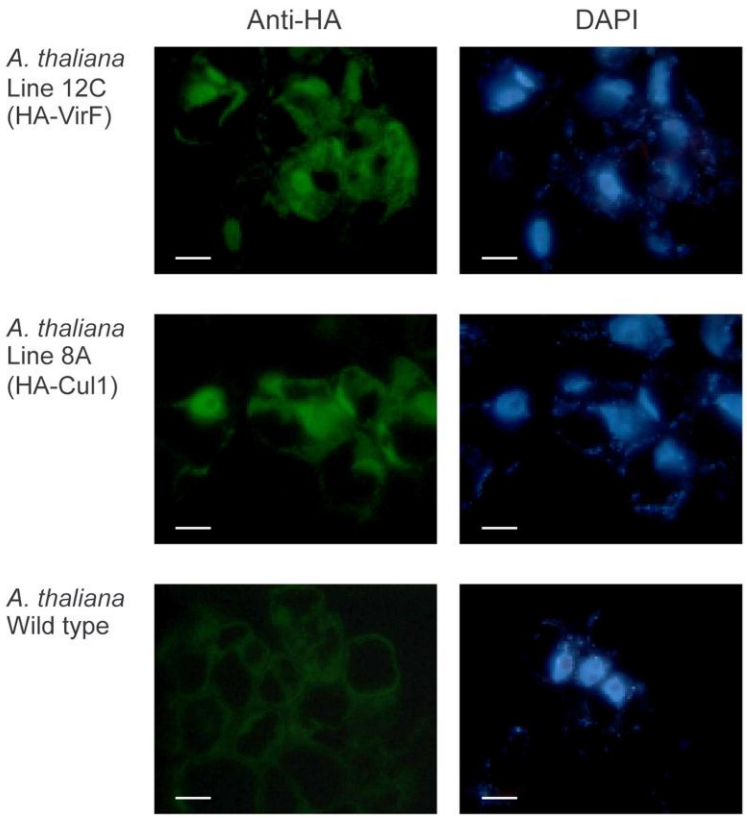

Figure 3. VirF localization in a cell line stably expressing VirF. Immunocytolocalization using HA antibodies showed that *A. thaliana* 12C cells expressing the HA-VirF protein in the nucleus and in the surrounding cytoplasmic region (green). Line, 8A, which expresses HA-Cul1, was used as positive HA control, and wild type cells, as negative control for HA expression. DAPI staining defines the location of the nuclei. Bar = 10 μM.

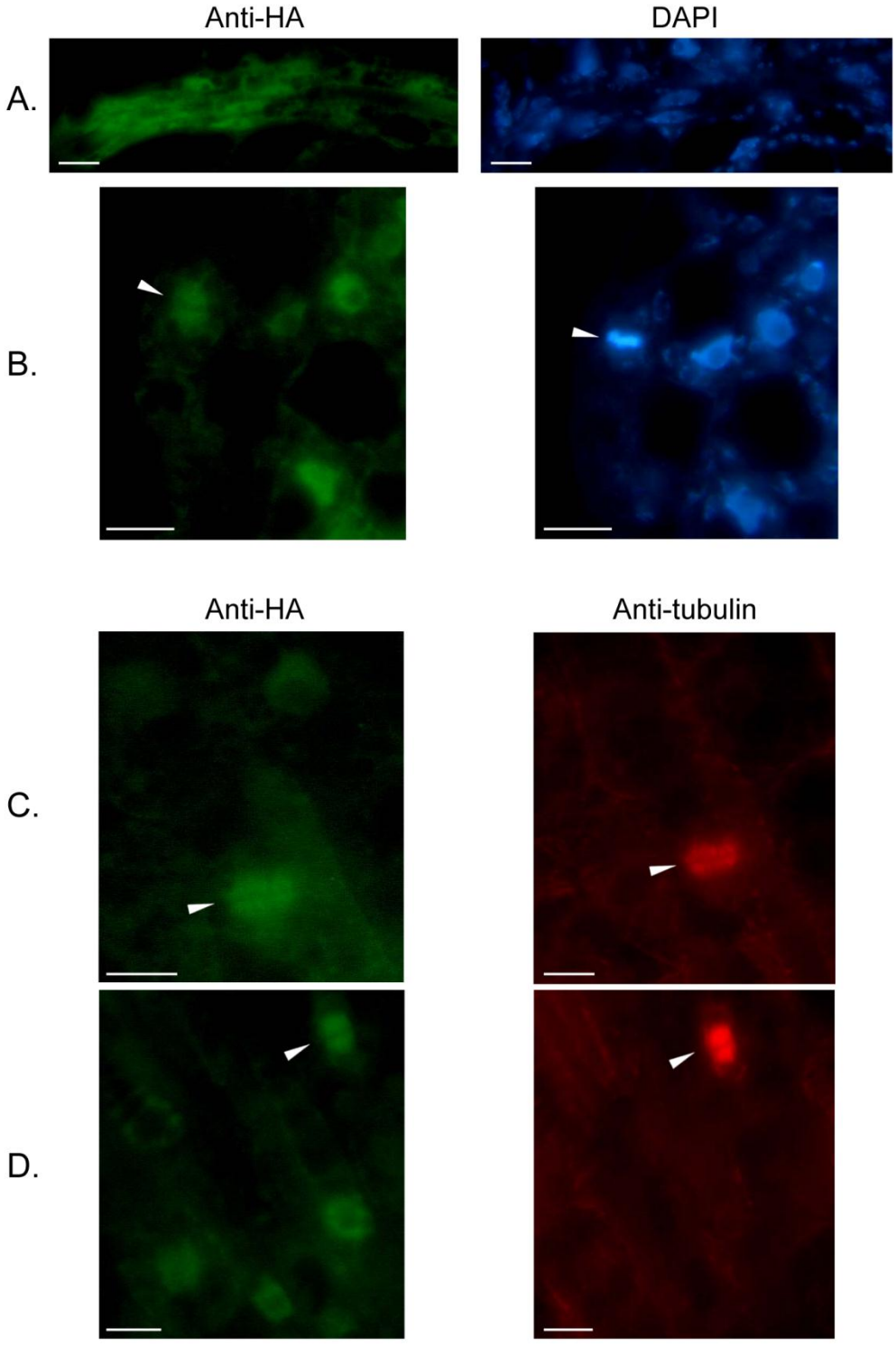

Figure 4. VirF localization in stably transformed plants expressing HA-VirF. Cells of *A. thaliana* seedlings (cotyledon) were used for immunocytolocalization of HA-VirF. **A)** Vascular tissue shows spread localization of VirF in nuclei and cytoplasm (green). DAPI staining defines location of the nuclei (blue). **B)** Cotyledon cells stained with anti-HA antibody and DAPI show VirF co-localizing with mitotic spindle. The upper left cell shows a dividing cell pattern (green), which marks the position of the mitotic spindle flanking the equatorially arranged DAPI-stained chromosomes (blue). **C)** Overlapping staining patterns of cells stained with anti-tubulin (red) and anti-HA antibodies (green) shows co-localization of VirF with phragmoplast. **D)** Co-localization of VirF with mitotic spindle showing overlapping staining pattern of anti-HA with anti-tubulin antibody. White arrows indicate the described structure. Bar= 5 μM.

Isolation of HA-VirF from *Arabidopsis* cells and preparation for co-imunoprecipitation assays

Since VirF was shown to be present in relatively large amounts in the line 12C expressing HA-VirF, we isolated the VirF protein from this line by immunoprecipitation and analyzed whether other protein subunits belonging to SCF complexes could be co-immunoprecipitated. VirF and $VirF_{(LP\text{-}AA)}$ expressing cultures (12C and 12D) were collected 17 weeks after transformation and submitted to protein extraction and Ion-Exchange Chromatography (IEC). Western blot analysis revealed that protein isolation from 5 g of suspension cells (line 12C, 2.6 μg/μl) gave an optimal yield of HA-VirF after elution with a NaCl gradient in the fraction of 200 mM (protein content of 0.8 μg/μl); however, HA-$VirF_{(LP\text{-}AA)}$ could not be isolated (Figure 5a and 5b), though the total protein content was around similar level (line 12D, 1.7 μg/μl) This was not unexpected as the $VirF_{(LP\text{-}AA)}$ mutant protein was expressed at a low level in the transformed cell line (12D). Immunoaffinity purification of proteins from the 200 mM NaCl IEC fraction using immobilized anti-HA IgG (Roche) led to recovery of HA-epitope-tagged VirF in the 500 mM NaCl eluate (Figure 5c), confirming our previous immunolocalization results. However, immunoanalysis using specific antibodies against other subunits of SCF complexes did not reveal additional proteins that co-precipitated with VirF.

Coimmunoprecipitation experiments and *in planta* interaction of VirF with members of the SCF complex

Though we succeeded in isolating VirF from whole cell extracts of VirF expressing cultures, we did not detect any other SCF protein in the precipitate. Therefore, to confirm the existence of an *in vivo* interaction of VirF with ASK1 (Schrammeijer *et al*., 2001) and to visualize the presence of additional SCF subunits in the same protein fraction, we modified the previous protocol in several steps. We worked at smaller scale but potentiated the efficiency of extraction and of total protein content by using a lower amount of PEX20 buffer per gram of tissue, faster and stronger cell disruption and an extra concentration procedure (Vivaspin column). Second, we eliminated the pre-clearing step with protein G used previously in the immunopurification of HA-VirF (see above) and tried the immobilization of HA antibodies on Affigel-10 agarose (BioRad Laboratories) instead of anti-HA IgG sepharose ready matrix (Roche). Affigel-10 couples proteins that become positively charged when pH buffer conditions are near or below their isoelectric point. In our study, coupling at or close to neutral pH helped to reduce binding of unspecific proteins to the affinity matrix. Thus, concentrated protein extract of HA-VirF expressing cells containing 3.0 mg of total protein was loaded onto the Affigel-10 agarose carrying immobilized anti-HA IgG. Also, since elution of HA-VirF was not possible with glycine 100 mM pH 2.5, strongly coupled proteins were dissociated instead with 8.0 M Urea. Our co-immunoprecipitation analysis of *A. thaliana* cell suspensions expressing a HA tagged VirF, revealed a weak signal for ASK1 coprecipitating in the same fraction with VirF (Figure 6A). In addition to this, we could observe that CUL1 also co-precipitated in the same fraction with VirF and ASK1, suggesting that these three subunits may form part of an SCF complex *in planta*.

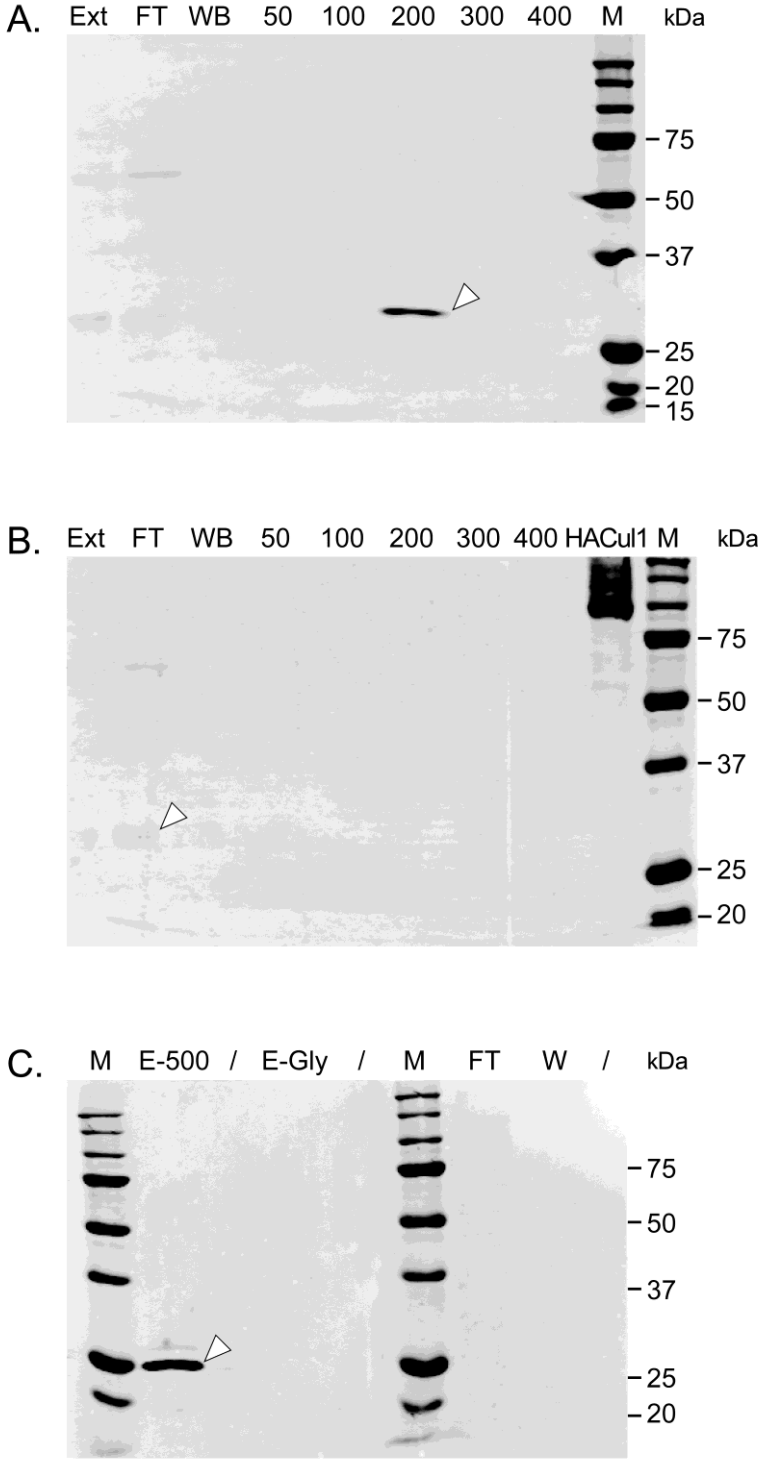

Figure 5. Isolation of VirF from suspension cells. VirF expressing cultures 12C **(A)** and 12D **(B)** were submitted to protein extraction and IEC. After elution with a 50, 100, 200, 300 and 400 mM NaCl gradient, western blot analysis revealed clear isolation of HA-VirF in the fraction eluted with 200mM NaCl **(A)** but the HA-VirF$_{(LP-AA)}$ protein was not recovered in this way **(B)**. Immunoaffinity purification of proteins contained in the mixed 100 mM and 200mM NaCl IEC fractions using immobilized anti-HA IgG (Roche Diagnostics Nederland, B.V.), showed recovery of HA-epitope-tagged VirF in the 500mM NaCl eluate **(C)**. Reading of the reaction was performed in an Odyssey infrared fluorescence scanner (LI-COR Biosciences). White arrows indicate the expected bands. M: protein standard BioRad marker, E-500: eluate by 500 mM NaCl, E-Gly: eluate by 100 mM glycine pH 2.5/100 mM Tris-HCl pH 8.0, FT: Flow-through and W: washing immunoprecipitation fractions.

Although we tried an immunoprecipitation reaction with the HA-tagged VirF$_{(LP-AA)}$ mutant protein (line 12D), the low expression of HA-VirF$_{(LP-AA)}$ made it difficult to draw any conclusion in relation to the binding of ASK1 or CUL1 to this protein. Nevertheless as controls, protein extracts derived from wild type cells and the line expressing HA-VirF$_{(LP-AA)}$ (12D) were immunoprecipitated and tested under similar conditions. Though some background of CUL1 is detected in the IP fraction as well as in unloaded wells, it is possible that this was due to sample run over, as no CUL1 was detected in the eluate fraction or in

the matrix after elution for the wild type extracts. It can be seen in Fig. 6a also that ASK1 and CUL1 did not bind aspecifically to the Affigel10 beads and thus that the presence of ASK1 and CUL1 in the IP of HA-VirF indicated their complex formation *in vivo*. In addition, the recovery of ASK1 in the urea eluate was highly increased by the addition of ATP during protein extraction and the immunoprecipitation reaction (Figure 6b). We could observe that in absence of ATP, ASK1 was retained strongly in the Affigel matrix together with the not eluted fraction of VirF, suggesting either a tighter interaction with VirF or larger protein availability when ATP was not present. The presence of ATP promotes allosteric changes in proteins that increase their dissociation from the ligand (Harlow and Lane, 1988). Though this is not clear for VirF and CUL1, it is possible that in our experiment this had a direct effect on the interaction of VirF and ASK, and thus on ASK1 elution. However, it may also be possible that additional interactions of ASK1 with proteins for which ATP may be required, for instance sucrose not fermenting related kinases (SnfRK, Farrás *et al*., 2001), reduce ASK1 availability for VirF binding. Unfortunately, we were not able to detect by immunoblot whether a related kinase (NPK) co-precipitated with VirF (data not shown). Additional studies thus are required to test whether ATP is involved in any possible phosphorylation reactions influencing the VirF-ASK1-CUL1 interaction.

In summary, here we show for first time that along with ASK1, plant CUL1 also co-precipitates with VirF and ASK1, shedding a new insight on the comprehension of the role of VirF as a member of a plant-assembled SCF complex.

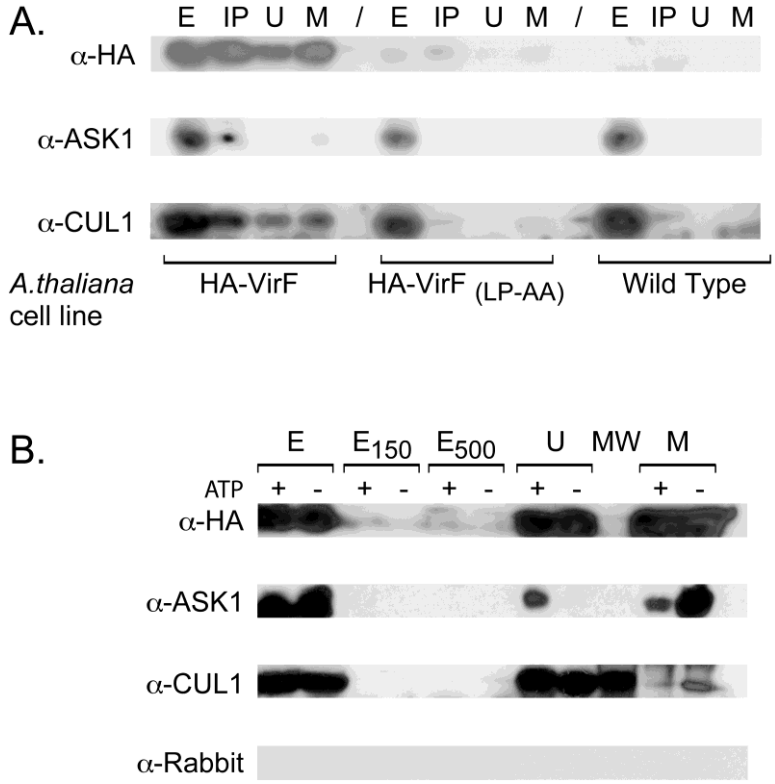

Figure 6. *In planta* protein interaction of VirF with ASK1 and CUL1. A) Western Blot analysis of anti-HA immunopre cipitates of HA-VirF expressing cell line (12C), HA-VirF$_{(LP-AA)}$ expressing cell line (12D) and wild type *A. thaliana* lines using anti-HA (α-HA), anti-ASK (α-ASK1) or anti-CUL1 (α-CUL1). Immunoblot detection with HRP conjugated rabbit antiserum (α-Rabbit) was used as negative control. E: plant extract, IP: immunoprecipitation reaction (non-eluted matrix), U: 8.0 M urea eluate, M: matrix after elution, /: no sample loaded **B)** Immunoblot detection of HA-VirF, ASK1 and CUL1 after HA-immunoprecipitation of line 12 C in presence or absence (-) of ATP.E: Plant Extract, E_{150}: eluate by 150 mM NaCl, E_{500}: eluate by 500 mM NaCl, U: eluate by 8.0 M Urea, M: matrix after elution, MW: protein standard BioRad marker.

DISCUSSION

VirF is a prokaryotic F-box protein (FBP) (Schrammeijer *et al.,* 2001) strongly conserved among *A. tumefaciens* octopine strains that is transferred to host cells via the T4SS (Vergunst *et al.,* 2000; Schrammeijer *et al.,* 2003). Complementation assays using *virF* mutant strains have shown the relevance of VirF to promote appropriate tumor growth after infection. Smaller tumors are formed on *Nicotiana glauca* plants after infection with *VirF* mutants of octopine strains and nopaline strains, which naturally lack a functional *virF* gene. This showed that strains lacking the *virF* gene cannot fully complete tumor formation in certain host plants and thus the virF protein plays a role as a host range determinant (Melchers *et al.,* 1990). Earlier studies suggested that VirF interacted with members of the SCF complexes and possibly played a role inside the plant cell during infection by mediating the recognition of target proteins for degradation. To confirm the interaction between VirF and ASK1/ASK2 proteins (Schrammeijer *et al.,* 2001), and also to detect whether other SCF proteins co-precipitate with VirF, we tried to express wild type and epitope-tagged versions of VirF in *A. thaliana* cell suspension cultures. Our study has validated the interaction of VirF with the ASK1 protein *in vivo* and has showed in addition that the CUL1 SCF subunit also co-immunoprecipitated with these two proteins.

First we tried to obtain cell lines co-expressing VirF in addition to ASK and/or Cullin epitope-tagged subunits to facilitate the detection of the co-precipitated proteins. Therefore, in addition to wild type *A. thaliana* cultures, we transformed stable ASK-myc and CUL-HA tagged expressing lines with different vectors carrying wild type and epitope-tagged *virF*. Three weeks after transformation with the *A. tumefaciens* strain LBA1100 (Beijersbergen *et al*., 1992), resultant cell lines showed progressive reduction of cell viability reflecting low transformation efficiency. Though the presence of the *virF* gene was detected in three cell lines, we were not able to detect the VirF protein in any of these cultures. We were able to obtain cell lines with improved cell-growth rate by using *A. tumefaciens* GV3101 (nopaline pMP90RK) (Koncz and Schell, 1986; Koncz *et al.,* 1994) for transformation. We detected the *virF* gene two weeks after transformation in a majority of these cell lines, and presence of the HA-v*irF* and HA-VirF$_{(LP\text{-}AA)}$ proteins 7 and 14 weeks after transformation. These findings showed the efficiency of the nopaline helper strain as appropriated helper to obtain sustainable transformed cell suspension cultures of *A. thaliana* (Ferrando *et al*., 2000).

Unfortunately, we were not able to detect expression of VirF in cultures previously transformed with ASK1 or Cullin tagged subunits (lines 7A, 7B, 7C 8A, 9A). From these, line 8A (CUL1-HA/T7-VirF) showed viability upon transformation, but though detection of CUL1-HA was appropriate and constant during the course of evaluation, immunodetection using the T7 epitope revealed a signal of 38 kDa that did not correspond to the size of the T7 –tagged VirF protein and was also present in wild type cells (data not shown). Reversely, though appropriate levels of the HA-VirF protein were detected in line 7C, the ASK-myc protein could no longer be detected in this line. It is known that transgenes introduced into higher plants can induce silencing of either homologous native genes -cosuppression- (Napoli *et al.,* 1990) or previously introduced transgenes -transinactivation- (Matzke and

Matzke, 1995) (reviewed in Furner *et al*., 1998; Rocha *et al*., 2005). In our experiments the use of similar expression modules (promoters, terminators) may have induced such trans-inactivation.

In transiently transformed cells, expression of VirF displays nuclear localization together with one of its proposed targets for degradation, VIP1 (Tzfira *et al*., 2004), though VirF itself appears not to contain any typical nuclear localization signal (NLS). Our immunolocalization analysis using lines 8A and 12C revealed that similar to HA-CUL1, HA-VirF was located in the nucleus as well as in the cytoplasmic region. It is known that the core proteins of the E3 ubiquitin ligases, ASK1, CUL1 and HRT1, are present both in the nucleus and in the cytoplasmic space (Blondel *et al*., 2000). In yeast, proteosomes are located near the endoplasmic reticulum (ER) (Enenkel *et al*., 1998), where their presence is required for the processing of protein precursors, but also in the outer or inner nuclear envelope (NE) (Wilkinson *et al*., 1998; Enenkel *et al*., 1998). Different FBPs display however distinct patterns of sub-cellular localization, depending mostly on the location of their substrates. Some FBPs are exclusively nuclear, (Cdc4, Far1) (i.e.), others however, can be found at both the NE and in the cytoplasm (Grr1; Blondel *et al*, 2000); or could present several isoforms that are active depending of their transient location (Fbw7; Ye *et al*., 2004) It is possible that after being translocated from the bacteria to the host cell, VirF travels to the nucleus in complex with a protein containing an NLS. During its journey to the plant cell nucleus, VirF may interact with ASK1 and CUL1 also in the cytoplasm and recognize targets (PIF) as protein precursors. However, further study is required to determine the co-localization of HA-VirF and PIF proteins and the direct role of VirF in the degradation of these predicted targets inside host cells.

Immunoprecipitation of HA-VirF led to co-precipitation of ASK1 and CUL1. We found that the yield of eluted ASK1 and CUL1 increased when ATP was present during the immunoprecipitation reaction of the HA-VirF line. Research by Farras *et al*. (2001), revealed an interaction of ASK1 with the *Arabidopsis* SnRK protein kinases and the alfa proteosomal subunit of the 20S core, suggesting the active phosphorylation of target proteins and a direct link between the SCF E3 ubiquitin ligase and the protein degradation machinery. The ATP effect seen in our immunoprecipitation needs much more study to find out about a possible phosphorylation reaction of targets in the presence of VirF as FBP. In the present study the use of specific anti-sera gave no clear indications for the presence of CUL1 interacting subunits CSN5 or RUB1 (Nedd8), nor of the 20S proteasome subunit and Snf1/AMPK related (SnRK) *Arabidopsis* protein kinases, in the immunoprecipitate of HA-VirF(data not shown).

Here, we confirm previous evidence of the *in vitro* interaction of VirF with ASK1 and ASK2 (Schrammeijer *et al.*, 2001), showing in addition that the CUL1 subunit co-precipitates together with VirF and ASK1. In summary the capacity of VirF to interact *in vivo* with the two main core proteins of a plant assembled SCF complex, ASK1 and CUL1, strongly supports our hypothesis that VirF plays a role *in planta* as FBP, most likely mediating the ubiquitination of proteins targeted for degradation via the proteasome.

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