CHAPTER 5

Evaluation of the stability of VirF interactors in yeast in the 
presence and absence of VirF

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ABSTRACT

The Agrobacterium tumefaciens VirF protein interacts with the Arabidopsis homologues of the 

yeast SKP1 protein, ASK1 and ASK2. This suggests that VirF plays a role inside the plant cell as part 
of a SCF complex recognizing target proteins for degradation. Earlier, we identified by yeast two hybrid 
analysis several proteins as putative interactors of VirF (PIF) and confirmed their interaction by in vitro 

binding assays. Here we analyzed whether VirF mediates the degradation of some of these putative 

interactors (VHA-B3, pirin-like and DAHP synthase 2 (known also as DHS2) in vivo, by using a protein 

degradation assay in yeast. To this end a gene encoding a histidine (His)-tagged VirF protein was 

cloned under the GAL1 inducible promoter and introduced into yeast cells expressing hemagglutinin 

(HA)-tagged PIF proteins constitutively. The expression level of VirF and the PIF proteins were 
determined after galactose induction of GAL1::virF, and at different time points after addition of 

glucose, which inhibits transcription of GAL1::virF. As a positive control for protein degradation we 

used the VirE2 Interacting Protein (VIP1), previously shown to be a target for VirF-mediated 

proteolysis in yeast. After total inhibition of de novo protein synthesis by cycloheximide (CHX), VIP1 

levels indeed decreased after VirF induction. In the absence of CHX, VIP1 levels initially also 

decreased after VirF synthesis, but then, in line with reduction of VirF expression, VIP1 rose to higher 

levels. Such VirF-dependent degradation pattern was seen weakly also for the VHA-B3 subunit, but 

not for pirin-like and DAHP/DHS2, which maintained stable expression levels either in the presence or 

absence of VirF. Our data suggest that VirF-dependent proteolysis of VHA-B3 by the 26S proteasome 

may be important in virulence of Agrobacterium tumefaciens on specific host plants.
INTRODUCTION

In eukaryotic organisms proteolysis plays a key role in regulating appropriate levels of proteins involved in different metabolic processes required for cell growth and development. The Ubiquitin-26S proteasome system (UPS) is currently the best described system responsible for the recognition and degradation of specific target proteins (reviewed by Lechner et al., 2006).

Ubiquitination of a substrate involves basically three enzymes known as Ubiquitin (Ub) activating enzyme (E1), Ub conjugating enzyme (E2) and Ub ligase enzyme (E3) (Figure 2 in Chapter 1). The E3-Ub ligases are formed by several subunits that associate to a core protein (Cullin), forming a multimeric complex that finally recruits the target protein for ubiquitination (reviewed by Welchman et al., 2005). Basically, these E3-Ub ligases can be classified in two main types: Homologous to E6-AP C-Terminus (HECT E3) and Really Interesting New Gene (RING) type E3 enzymes. The SCF complex is a RING-E3 Ub ligase that catalyzes the direct transfer of an Ub molecule from the E2-Ub conjugating enzyme to the E3-Ub ligase bound substrate. It is composed of at least four subunits: S-phase-kinase associated protein/suppressor of kinethochore protein (SKP1), Cullin (CUL1), RING-BOX1 (RBX1)/Regulator of Cullins (ROC1) and a F-box protein (FBP), the latter protein being the substrate recognition protein for degradation (reviewed by Cardozo and Pagano, 2004; Angot et al., 2007). Though the presence of around 20 genes encoding FBPs in the Saccharomyces cerevisiae genome (Hermand, 2006) gives a certain degree of diversity of SCF complexes, in plants and animals high numbers of other SCF subunit homologs (>20 SKP1 homologs in Arabidopsis) create an even higher degree of possibilities for specific regulation of protein degradation (Risseeuw et al., 2003). Two main amino acid (aa) regions characterize most FBPs: an N-terminal F-box domain that directly interacts with the SKP1 subunit and mediates the interaction between the FBP and the E3-ligase core, and a protein-protein interaction domain responsible for target protein recognition. Most FBPs carry this second interaction domain at the C-terminus, which can be either a WD-repeat domain (FBWs), a LLR (leucine-rich repeat) (FBLs) or another protein-protein interaction domain (FBXs) (Cardozo and Pagano, 2004).

Studies of the infection process of the plant pathogen Agrobacterium tumefaciens have led to the identification of several virulence factors produced by this bacterium, which have an important function during the course of infection. Attempts to elucidate the role of some of the virulence factors such as VirD2, VirE2, VirE3 and VirF during the infection process have shown the importance of their interaction with host (plant) proteins to promote T-DNA nuclear uptake and favor the transformation process (Ballas and Citovsky, 1997; Bakó et al., 2003; Deng et al., 1998; Tzfira and Citovsky, 2001; Schrammeijer et al., 2001; Tzfira et al., 2004; García-Rodríguez, Schrammeijer and Hooykaas, 2006). Once translocated inside the plant cell, the VirE2 protein associates with the T-strand to ensure an optimal journey through the host cell cytosol of the so-called T-complex. Interaction of the nuclear localization signal (NLS) of VirD2 with importin-α guarantees the efficient nuclear uptake of the T-DNA, while interaction of VirE2 with two plant proteins, VIP1 and VIP2, further facilitates the transport of the T-DNA into the host cell nucleus (Tzfira and Citovsky, 2001). The VirF protein is known to
interact with the *Arabidopsis* homologues of the yeast SKP1 protein, ASK1 and ASK2, via an N-terminal F-box domain. Therefore it was suggested that VirF may form part of an SCF complex in the host and bind specific proteins for targeted degradation by the 26S proteasome (Schrammeijer *et al*., 2001).

Earlier, we identified several putative VirF interacting (PIF) proteins from plants (Chapter 3). Although *in vitro* pull-down assays confirmed interactions of several candidate substrate proteins, the VirF mediated degradation of these proteins, as well as the relevance of these interactions during infection, still needs to be confirmed *in vivo*. In the present work, we analyzed the VirF-mediated degradation of these plant proteins *in vivo*. To this end, we used a protein degradation assay in yeast by co-expressing VirF with some of its putative interactors or PIF proteins (Chapter 3). Using a similar strategy in yeast, Tzfira *et al*., (2004) showed the VirF-mediated degradation of the VirE2 interacting protein VIP1, and through VIP1, indirectly of VirE2. Here, we obtained further evidence for targeted degradation of one of the identified VirF interactors, the VHA-B3 subunit.

**EXPERIMENTAL PROCEDURES**

**VirF - SKP1 Interaction assay**

To determine whether VirF interacted with the yeast SKP1 protein, we performed a GST pull down assay using tagged versions of VirF (Glutathione S-transferase, GST) and SKP1 (6X-Histidine, HIS). To obtain the HIS::SKP1 fusion, primers SKP1-1 (5'-CCctcgagGTGACTTCTAATGTTGTCC-3', *Xho*I underlined) and SKP1-2 (5'-GGggtaccCTAACGGTCTTCAGCCC-3', *Kpn*I underlined) were used to amplify the SKP1 gene using pJS96 (Schouten, 1999) as template. The PCR product was cloned as *Xho*I-*Kpn*I in the pET16H expression vector (Novagen) giving origin to pSDM3034. Construction of the GST::virF fusion (pSDM3514) in the pGEX-KG vector (Guan and Dixon, 1991) is described in Chapter 3 of this thesis. Plasmids were used to transform *Escherichia coli* BL21-DE3 strain (Novagen) and cells were submitted to IPTG induction. Additional experimental conditions to perform a GST pull-down assay with the heterologously expressed proteins were the same as described in Chapter 3. Detection of His-tagged SKP1 was performed by Western blot analysis using penta-His antibodies (1:2000 dilution) (Qiagen, Benelux B.V.).

**Construction of yeast expression vectors**

To obtain inducible expression of His-VirF fusion protein, a *BamH*I-*Not*I virF fragment was obtained from pBI771::virF∆ATG (Schrammeijer *et al*., 2001) and cloned into pMVHis (2mu; *URA3*) (van Hemert *et al*., 2003) plasmid, allowing the galactose-inducible expression of His6-tagged proteins in yeast. This gave origin to pSDM3539. To express the putative targets of VirF or PIFs constitutively (Chapter 3), we cloned the coding sequences of some of the PIFs into the low copy number p415GPD vector (CEN6/ARSH4; *LEU2*) (Mumberg, Müller and Funk, 1995). To this end, two complementary oligonucleotides encoding two peptide chains for the HA epitope were synthesized containing sticky ends (underlined) for *Xba*I and *BamH*I (5'-ctagaAAATGGAATATCCATATGATGTTCCAGATTATGCTGGATATCCATATGAT
GTTCGAGATTATGCTA-3'). This linker was inserted after the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter region of the p415GPD expression vector, giving origin to pSDM3531. Subsequently, to obtain the HA tagged fusion proteins, the coding sequences of the PIF proteins were amplified using as template plasmid DNA of the pET16H vectors carrying these genes (Chapter 3). Correctness of the sequences of the resulting plasmid pSDM3531 and its derivatives was confirmed by DNA sequencing (Base Clear Laboratories, The Netherlands). Oligonucleotide sequences, restriction sites and pSDM numbers for templates and final resulting plasmids are described in Table1.

<table>
<thead>
<tr>
<th>PIF*</th>
<th>Template DNA</th>
<th>Primers**</th>
<th>Restriction sites</th>
<th>Final pSDM number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (DAHP)</td>
<td>pSDM3523</td>
<td>F: 5'-AAAactcgtTGTTGACTCTAAA CGCTTCTCC-3'</td>
<td>PstI XhoI</td>
<td>pSDM3538</td>
</tr>
<tr>
<td>2 (Lon protease-like)</td>
<td>pSDM3517</td>
<td>F: 5'-CCCagcgtGGCCCTTCCACAC GTATGC-3'</td>
<td>HindIII XhoI</td>
<td>pSDM3535</td>
</tr>
<tr>
<td>3 (pirin-like)</td>
<td>pSDM3525</td>
<td>F: 5'-TCCcccgggGTGTTCTTCTTAC TTCCAAAG-3'</td>
<td>SnaI XhoI</td>
<td>pSDM3534</td>
</tr>
<tr>
<td>4 (VHA-B3)</td>
<td>pSDM3526</td>
<td>F: 5'-AAAactcgtTGTTGGAGACTAG TATCGAC-3'</td>
<td>HindIII PstI</td>
<td>pSDM3537</td>
</tr>
<tr>
<td>JAIP</td>
<td>pSDM3529</td>
<td>F: 5'-AAAactcgtAGTGACCGCTGGA GAACTAAGATC-3' R: 5'-CCGctcgAGTTAGCTGAATTG ACCGCATAG-3'</td>
<td>PstI XhoI</td>
<td>pSDM3536</td>
</tr>
</tbody>
</table>

* DAHP: 3-deoxy-D-arabino-heptulosonate 7-phosphate or DHS2
VHA-B3: vacuolar H+ ATPase subunit B3
JAIP: jasmonic acid inducible protein or epithiospecifier gene
**F: Forward
R: Reverse

For cloning of the VirE2 interacting protein (vip1) gene, the full coding sequence (cds) was amplified using pSDM3268 DNA (C. Michielse, unpublished) as template with primer V1P1-3, carrying a Xmal site (underlined) (5'-TCCcccgggAAGGAGAGGAAGAGGA-3') and primer V1P1-4 (XhoI underlined) (5'-CCGctcgAGTTAAGCTGAATTG ACCGCATAG-3'). The PCR product was finally cloned as an N-terminal HA tagged fusion in pSDM3531 (Xmal-XhoI), resulting in pSDM3532.
Transformation of yeast cells and protein expression assay

A five ml volume of an overnight culture of *Saccharomyces cerevisiae* GG0423 (CEN-PK102-3A [MATα ura3-52 leu2-112 TRP-289 HIS-delta1]; M. Meger, Utrecht, The Netherlands) was used to inoculate 100 ml of YPD medium (Sherman, 1991). After four hours incubation (30°C, 150 rpm), cells were pelleted (2500 rpm) in aliquots of 50 ml and washed with 25 ml and 1 ml of Milli-Q (MQ) water (Millipore). According to Gietz and Woods (2002), 5 μl of purified pSDM plasmid (Qiagen) containing the final HA::PIF fusion or *HIS::virF* (pSDM3539), were added to a transformation mix containing 240 μl 50% Polyethylene glycol (PEG), 36 μl of 1 M LiAc, 40 μg of carrier DNA (Yeastmaker™[10mg/ml], Clontech Laboratories, Inc. Palo Alto, CA) in a final 300 μl volume. This preparation was added to 50 μl of yeast cells, which after 30 min incubation at 30°C were submitted to heat shock (20 min at 42°C, ice cold 5 min). Cells were pelleted at maximum speed (13000 rpm) for 30 sec and resuspended in 300 μl MQ water. *LEU2* (p415GPD derivatives Table 1 and pSDM3532) and *URA3* (pMVHis) were used as selectable markers after plating 150 μl for each transformation on Synthetic Dropout minimal medium (SD: 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, 1.5% bacto-agar), supplemented with leucine (Leu 0.03 g/l), for pMVHis::VirF (pSDM3539), or uracil (Ura 0.02 g/l), for the *HA::PIF* fusions (pSDM3534-3538). After three days incubation (30°C), individual colonies were inoculated in 1 ml of similar SD medium and placed overnight at 30°C (120 rpm). For each culture, 0.8 ml were pelleted (30 sec, 13000 rpm) and resuspended in 25 μl of MQ water to be finally mixed with 25 μl of 2X SDS-PAGE loading buffer and analyzed by 11% acrylamide gel electrophoresis. Samples were analyzed by immunoblotting (Chapter 3) with monoclonal horseradish peroxidase (HRP) conjugated HA antibodies (anti-HA-HRP conjugate, 1:1000; Roche Diagnostics Nederland B.V) for detection of HA tagged PIF fusions, or with penta-His antibodies (Qiagen, Benelux B.V.; 1:2000 dilution), to determine the presence of VirF. Tagged proteins were blotted onto a polyvinylidene fluoride (PVDF)-Immobilon P-Millipore membrane and detected by chemiluminiscence after addition of HRP substrate (Lumiglu reagent-Cell Signaling Technology, Westburg, The Netherlands).

Protein degradation assay

*S. cerevisiae* GG0423 cells able to express *HIS::virF* after galactose induction (pSDM3539), as well as cells carrying empty pMVHis vector, were co-transformed with a specific *HA::PIF* fusion vector previously tested for expression (pSDM3532, 3534, 3537 or 3538) using the protocol described above. From a 7 ml overnight culture in SD (-Leu/-Ura) of each co-transformed strain, 100 ml of SD (-Leu/-Ura were inoculated to an (optical density (OD600= 0.1) and incubated to obtain an OD600 between 0.4 and 0.5. Aliquots of 50 ml were pelleted (10 min, 4000 rpm), washed once with 50 ml of medium lacking glucose ( SD (-Leu/-Ura/-glu) and resuspended in 5 ml of this last medium to obtain a 10-times (10X) OD600 cell suspension (4 to 5 OD Units/ml). Finally, using SD (-Leu/-Ura/glu) supplemented with 2% galactose, three aliquots of 50 ml were prepared using the last preparation with an initial OD600 of 0.3 both for cultures of pSDM3539 (pMVHis::virF) as well as for pMVHis (HIS
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control) previously cotransformed with the respective HA::PIF or HA::VIP1 fusion. After overnight induction (30°C, 150 rpm), each culture and the respective control were supplemented with glucose (2%) in absence or presence of cycloheximide (CHX, 50 µg/ml), or after elimination of residual galactose by centrifugation (10 min, 4000 rpm). Samples of 2.5 ml were pelleted and resuspended in a final volume (µl) equivalent to 50X their OD<sub>600</sub> value of 1.5X SDS-PAGE sample buffer. We analyzed different time points (T) starting at the moment of galactose induction (T= 00), glucose addition with or without CHX/centrifugation (T= 0) and subsequent hour intervals (T= 0.5, 1.0, 1.5, 2.0, 2.5, 3.5 and 4.5/5.0 hours) by loading 8 µl per sample (0.16 OD<sub>600</sub>) on an 11% SDS-PAGE gel. To determine equality of the cell extract volume loaded onto the gels, as well as protein instability during the different time points due to other factors than VirF, we applied Coomassie blue staining (Sambrook, Fritsch and Maniatis, 1989) and performed immunoblot detection of the highly stable yeast protein Bmh1 (Brain Modulosignalin Homologue, UniprotKB/Swiss-Prot entry P29311; anti-Bmh1: 1:2000). Considering the uniformity of the protein levels among samples observed by Coomassie blue staining, we assumed equality of the cell extract volume into the gels. Analysis of protein expression both of HIS::VirF as well as the HA::PIF fusions was performed by immunodetection by Western blot analysis as described above.

RESULTS AND DISCUSSION

Like animals and plants, yeast cells employ SCF complexes to instruct the targeted degradation of proteins by the proteasome (Patton, Willems and Tyers, 1998; Spielewoy et al., 2004; Willems et al., 2004; Brunson et al., 2005). In yeast, heterologous proteins can be easily expressed either constitutively, or using inducible or repressible systems. Therefore we have used this model organism to study whether the FBP VirF could mediate the targeted degradation of several of the putative interactors of VirF (PIF) identified in Chapter 3. To this end, the protein levels of epitope-tagged versions of the VIP1 protein and some of the identified putative VirF targets (Chapter 3) were compared in both the presence and absence of VirF.

Before embarking on these experiments, we first tested whether VirF interacts with the yeast SKP1 protein. By using a GST pull down assay, we were indeed able to find evidence for a direct interaction of VirF with the yeast SKP1 protein (data not shown), as this was previously observed for the plant homologue ASK1 (Schrammeijer et al., 2001). This suggested that VirF could form an SCF complex in yeast, and consequently, would be able to present targets for degradation to the yeast proteasome system.

To compare protein levels of the respective target proteins in both the presence and in the absence of His-VirF, and thus the stability of these targets, we constructed yeast strains with two plasmids: one expressing His-VirF (or His alone, as control in the absence of VirF) from a galactose inducible promoter, and a second plasmid with the respective HA-tagged PIF under control of a constitutive GPD promoter. The expression level of VirF and the putative interacting proteins was determined before and after galactose induction of GAL1::virF, and at several time points after addition of glucose, which inhibits transcription of GAL1::virF. As a positive control for protein degradation we used the VirE2 Interacting
Protein VIP1, previously shown to be a target for VirF-mediated proteolysis in yeast (Tzfira et al., 2004). Protein levels of both VirF and PIFs were determined using Western blot analysis. Unfortunately, after several attempts the Lon protease like (expressed from plasmid pSDM3535) and the JAIP (expressed from pSDM3536) proteins were not expressed in yeast cells and therefore could not be analyzed. It is possible that precise post-translational modifications that do not occur in yeast are required for proper folding and function of these plant proteins. However, the HA-tagged versions of the pirin-like (pSDM3534), VHA-B3 (pSDM3537) and DAHP/DHS2 (pSDM3538) proteins could be detected on Western blots (data not shown), and also VirF expression was detected in yeast lines containing Gal1::virF (pSDM3539) after induction by galactose.

Studies by Tzfira et al., (2004) had employed a similar yeast system and had revealed that VirF recognized VIP1, and through VIP1, its binding partner VirE2, are targeted for degradation via the 26S proteasome. We therefore decided to determine if VIP1 expression levels were similarly affected by induction of VirF in our system and we introduced pSDM3532 encoding HA-tagged VIP1 into yeast carrying Gal1::virF (pSDM3539). Two independent transformants (clones 12 and 16) were tested. Galactose was added to the cultures to induce expression of virF and indeed after overnight (24 hours) incubation induction of VirF was seen (compare T= 00 before addition of galactose with T= 0 after 24 hours incubation with galactose). At the same time it can be seen that the level of VIP1 is much higher at T= 00 as compared to that at T= 0, suggesting that VIP1 becomes unstable in the presence of VirF.

To see if the levels of VIP1 increase when VirF disappears (Figure 1) again from the cell, glucose was added to the cultures at T = 0, which leads to repression of transcription of the Gal::virF gene. As a control we added in Figure 1A also at the same time the translation inhibitor cycloheximide (CHX). As can be seen in Figure 1B, the levels of VIP1 increase again when VirF disappears from the cell two hours after the addition of glucose. In the control (Figure 1A) no increase of VIP1 levels can be seen after the disappearance of VirF in the presence of CHX, which also may have an effect on the de novo synthesis of VIP1.

Comparison of Figures 1A and 1B indicates that VirF disappears more rapidly from the cells in the presence of the translation inhibitor CHX. This might suggest an increased instability of VirF in the presence of CHX or a lack of continued VirF synthesis in the presence of CHX. Besides, comparing the higher level of VIP1 in similarly treated control cells carrying a wild type pMVHIs with the lower level found in the tested VirF transformed cells, it is evident that the presence of Gal1::virF, even before induction, remarkably affects the basal expression levels of VIP1. Constitutively expressed BMH1 levels are invariant in the control and in the tested cells, suggesting that the sharp fluctuation of VIP1 levels could in fact correlate with the presence of VirF.

In order to discriminate between these possibilities a third experiment (Figure 1C) was done in which glucose was added and no CHX, but in which the cells were washed in order to remove any residual inducing galactose. As can be seen in Figure 1C, washing away of galactose leads to a much more rapid disappearance of VirF from the cells than in the absence of washing, indicating that the continued presence of VirF in the experiment shown
Figure 1. Immunoblot detection of VirF mediated VIP1 protein degradation. Comparison of constitutive HA-VIP1 and induced His-VirF after overnight galactose activation of GAL1::HIS::virF (pMVHis::VirF + VIP), followed by glucose repression in the presence (A) or absence (B) of CHX and after elimination of residual galactose by centrifugation in absence of CHX (C). Proteins were identified by Western blot analysis using antibodies against His-tag (α-His), HA-tag (α-HA) or BMH1 (α-BMH1) before galactose addition (time= 00), after overnight induction (time= 0) and at different time points after glucose repression/CHX addition (time= 0.5 to 5 hours). Protein isolates from cultures lacking VirF (pMVHis + VIP) were used as control. Signal intensity of VIP1 is correlated with expression of intrinsic BMH1. **Gal**: galactose, **Gluc**: glucose, **CHX**: cycloheximide Twisted arrow indicates centrifugation.
in Figure 1B was due to prolonged synthesis due to residual galactose inducer. In the controls in which yeast cells lacking the \textit{Gal1::virF} construct were treated in the same way, also somewhat lower levels of VIP1 were seen after 24 hours incubation in galactose. This small negative effect may be due to a small difference in activity of the \textit{GPD} promoter depending on the carbon source (Frugier, Rijckelinck and Giegé, 2005). As a general conclusion then we could affirm that the levels of VIP1 are higher in the absence than in the presence of the \textit{virF} gene (similar conditions and exposure times were used). This may indicate that although not visible on the Western blot, some VirF is present already under non-inducing conditions, resulting in some degradation of VIP1, even before induction of VirF with galactose (indicating slight leakiness of the gal-inducible promoter). This, however, strengthens the conclusion that the disappearance of VIP1 is VirF-dependent.

**Figure 2.** Protein degradation assay for PIFs. Protein degradation pattern for constitutive HA-tagged PIF proteins VHA-B3 subunit, pirin-like and DAHP (DHS2-synthase) after overnight galactose activation of \textit{GAL1::His::virF} (pMVHis::VirF), followed by glucose repression after elimination of residual galactose by centrifugation in absence of CHX (left panel). Proteins were identified by western blot analysis using antibodies against His-tag (\(\alpha\)-His), HA-tag (\(\alpha\)-HA) or BMH1 (\(\alpha\)-BMH1) before galactose addition (time= 00), after overnight induction (time= 0) and at different time points (time= 0.5 to 4.5 hours) after glucose repression. Protein isolates from cotransformed cultures lacking VirF (pMVHis) were used as control (right panel). Signal intensity of VIP1 is correlated with expression of intrinsic BMH1. \textbf{Gal}: galactose, \textbf{Gluc}: glucose. Twisted arrow indicates centrifugation.
In summary, our experimental results are in line with VirF dependent degradation of VIP1 in yeast. Therefore, we performed similar experiments with the previously identified interactors of VirF or PIF proteins (Chapter 3). We used the conditions described for the third series of experiments (Figure 1C) for these experiments. The results (Figure 2) indicated that the levels of the VHA-B3 subunit may be slightly affected by the presence of VirF, but no effect at all is seen on pirin-like or DHS2 stability. This suggests that from these three proteins, VirF could possibly only destabilize the VHA-B3 subunit in a similar way as VIP1 in yeast.

The results suggest that pirin-like and DHS2 may not be true in vivo interactors of VirF, although they interact in the yeast two-hybrid assay (Chapter 3) and in pull down in vitro experiments. This may be due to a different localization of VirF and the PIFs in the yeast cell in vivo. Further analysis needs to be done in the natural host plant cells to see whether these proteins can interact in vivo in plant cells (for instance by bimolecular fluorescence complementation assays or co-precipitation experiments) and are real targets for VirF-mediated degradation.

Recent studies suggest that virulence proteins from a diversity of pathogens, i.e. from viral (Pazhouhandeh et al., 2006) or prokaryotic origin (Shigella flexneri [OspG], Kim et al., 2005; Pseudomonas syringae [AvrPtoB], Abramovitch et al., 2006, Janjusevic et al., 2006; Ralstonia solanacearum [GALA], Angot et al., 2006; and others (reviewed by Angot et al., 2007) interfere in different ways with the ubiquitination system of the eukaryotic host cell. Some bacterial effectors show intrinsic E3 ubiquitin ligase activity once translocated into host cells, as is the case for AvrPtoB from Pseudomonas syringae pv tomato, which is able to ubiquitinate Fen, a kinase related to Pto proteins found in resistant tomato varieties, and promote its degradation in a proteasome dependent manner (Rosebrock et al., 2007). These studies point to a co-evolution of pathogens and their hosts and show how prokaryotic pathogens evolved to interfere with specific eukaryotic systems to adapt host cell biology to their advantage and colonize the host. In the present study, further evidence was obtained that Agrobacterium tumefaciens uses such strategies during the infection process.

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REFERENCES


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