CHAPTER 3

Isolation of *Arabidopsis* proteins that interact with the *Agrobacterium* virulence protein VirF

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ABSTRACT

The *Agrobacterium tumefaciens* VirF protein is directly translocated into host cells via the VirB/VirD4 transport system during the infection process. It is a host range determinant required for full virulence on certain host plants, and absence of *virF* in *Agrobacterium* leads to attenuated virulence on such hosts but can be complemented by *in planta* expression of VirF. The precise function of VirF in the host cell is not clear but VirF contains an F-box domain by which it interacts with the *Arabidopsis thaliana* homologues of the yeast SKP1 protein (ASK1 and ASK2). SKP1 and F-box proteins (FBPs) are subunits of the SCF-Ubiquitin protein ligases (E3 ligases) that play an essential role in many cellular processes by recruiting specific proteins for ubiquitination and subsequent degradation by the 26S proteasome. F-box proteins are the key element of the SCF-complex, as they are responsible for the recognition of the proteins to be degraded. To identify target proteins of VirF we performed a yeast two-hybrid screen with VirF as bait using an *A. thaliana* cDNA library. Putative interactors of VirF (PIFs) were identified; GST pull down assays confirmed for five selected proteins direct binding to VirF *in vitro*. These five PIFs represent a Lon protease like, a putative pirin like protein, 3-deoxy-7-phosphoheptulonate 7-phosphate (DAHP) synthase 2 (DHS2), the vacuolar H+ ATPase subunit B3 (VHA-B3) and branched-chain amino-transferase (BCAT4). VirF may enhance virulence by stimulating the targeted degradation of some or all of these proteins. How this could promote virulence is being discussed.
INTRODUCTION

The capacity of Agrobacterium tumefaciens to induce tumorigenesis in a wide range of host plants involves the translocation of a segment of oncogenic DNA called the transferred DNA (T-DNA) and several effector or virulence proteins, which are encoded by genes located in the vir-region on the Ti plasmid, into host cells. Specific phenolic compounds, such as acetosyringone, which are released upon wounding of plant cells, activate the VirA/VirG two-component regulatory system that subsequently induces the expression of the vir genes (Reviewed by Hansen and Chilton, 1999; Gelvin, 2003; 2006; McCullen and Binns, 2006). Proteins encoded by the virB and virD4 genes form a transport apparatus, also called type IV secretion system (T4SS), that spans the bacterial envelope and through which protein and DNA are transferred from the bacterium into the plant cell (Christie, 2004). The T-DNA is derived from the T-region of the Ti-plasmid, which is flanked by an imperfect 24 bp direct DNA repeat. The VirD2 protein introduces a nick at the border repeats and possibly via a replacement mechanism a single stranded form of the T-region called T-strand is released. VirD2 remains attached to the 5′ of the T-strand, and pilots the T-strand through the T4SS into the host cell (reviewed by Hansen and Chilton, 1999; Gelvin 2003). The VirE2 protein is an effector protein transferred into host cells (Vergunst et al., 2000; Schrammeijer et al., 2001), where it binds to the T-strand, protecting the T-strand against nucleolytic attack (Ziemienowicz et al., 2001). By the presence of nuclear localization signals (NLS) both in VirD2 and VirE2, and interactions of these proteins with plant proteins, the T-complex is directed to the cell nucleus where it becomes integrated in the host genome (Zambryski, 1992; Ballas and Citovsky, 1997; Pelczar et al., 2004).

Use of the Cre reporter assay for protein translocation (CRAFT) directly demonstrated transfer of the effector proteins VirF, VirE2, VirE3, VirD2 and VirD5 to plant and yeast cells via the VirB/VirD4 channel even in absence of T-DNA transfer (Vergunst et al., 2000; Schrammeijer et al., 2003; Vergunst et al., 2003; Vergunst et al., 2005; Chapter 2). Although the function of VirE2 and VirD2 in planta has been clarified to a large extent, the function of additional translocated virulence proteins such as VirF, VirE3 and VirD5 has not been clearly defined yet. Previous extracellular complementation tests demonstrated that the attenuated virulence of virF mutant strains on N. glauca was restored by co-infection with A. tumefaciens helper strains, lacking the T region but expressing the VirF protein (Otten et al., 1984). Full virulence of the virF mutant was also seen after infection of N. glauca plants expressing the VirF protein (Regensburg-Tuïnk and Hooykaas, 1993). These data suggested that VirF has a function inside the plant host. Like VirF, VirE3 is a host range factor; although a virE3 deletion only has a minor effect on virulence on specific host plants, it dramatically aggravates the effect of a virF mutation. (Schrammeijer, 2001, García-Rodríguez, Schrammeijer and Hooykaas, 2006). Later findings showed that VirE3 interacts with specific plant nuclear proteins, and it was suggested that VirE3 may be a transcriptional activator of genes involved in tumor formation (Garcia-Rodríguez, Schrammeijer and Hooykaas, 2006). On the other hand VirE3 was shown to interact with VirE2 and it was suggested that VirE3 could fulfill a task similar to that of the plant VirE2 interacting protein (VIP1), mediating the import of VirE2 into the nucleus (Lacroix et al., 2005).
It was shown that the *A. tumefaciens* VirF protein interacted with the *Arabidopsis thaliana* SKP1 homologues ASK1 and ASK2 (Schrammeijer *et al.*, 2001). The SKP1 proteins of yeast and other eukaryotes form part of so called SKP-Cullin-F-box protein (SCF) complexes, which have E3 ubiquitin (Ub) ligase activity for proteins that are destined for degradation by the proteasome. F-box proteins (FBPs), which bind to SKP1 and thus to the SCF complex through their F-box, are responsible for the recruitment of specific proteins to the complex that are to be degraded. Further research taught that VirF has an F-box that is essential for its function (Schrammeijer *et al.*, 2001). The conserved F box residues leucine and proline were shown to be important for this interaction, and also for virulence (Schrammeijer *et al.*, 2001; Chapter 6). The presence of an F-box in VirF suggested a role for VirF in recruitment of specific host proteins that inhibit or reduce bacterial virulence, followed by their degradation by the 26S proteasome. Although a clear function for VirF has not been determined yet, Tzfira *et al.* (2004) suggested that VirF plays a role in uncoating of the T-strand prior to integration in the host genome by targeting the VirE2 interacting protein VIP1, and thereby indirectly VirE2, for degradation.

Here we used the yeast two-hybrid assay with VirF as a bait to identify proteins that may be targets for proteolysis during *A. tumefaciens* infection. Previously, a screen with full length VirF resulted in isolation of host proteins interacting specifically with the F box domain of VirF, ASK1 and ASK2 (Schrammeijer *et al.*, 2001). FBPs have in their C-terminal domains protein-protein interaction domains (WD40, LRR) by which substrate proteins are bound. However, not all FBPs, including VirF, contain such an obvious domain. In order to preferentially identify target proteins and avoid isolation of ASK proteins, we used a VirF protein devoid of its F-box as a bait to screen an *A. thaliana* cDNA library. This resulted in the isolation of several potential target proteins. By *in vitro* GST pull-down assays we further confirmed direct binding of five PIFs to VirF. Further work needs to establish whether VirF induces the targeted degradation of these proteins, and how this enhances crown gall tumor formation.

**EXPERIMENTAL PROCEDURES**

**Construction of bait plasmids**

Three different versions of the *virF* gene (*Schrammeijer, et al.*, 2001) were fused to the *GAL4*-DNA binding domain (*GAL4BD*) in the high copy number pAS2-1 bait vector (Clontech Laboratories, Inc. Palo Alto, CA). The first fusion was constructed with the complete coding sequence (CDS) of *virF* but devoid of its start codon (*virF*ΔATG); the second fusion had a deletion of the 5′- 126 nucleotides, encoding the F-box (*virF*Δ42N); the third version contained mutations resulting in amino acid changes of the conserved leucine and proline (LP) residues in the F-box for alanine (A) (*virF*ΔATG<sub>(LP to AA)</sub>) (Schrammeijer *et al.*, 2001). A palindromic linker containing a NotI site (5′-GGCGGGCCGCTGCA-3′) was previously introduced between *SalI* and *PstI* of the pAS2.1 plasmid. Then pBl770::*virF*ΔATG, pBl770::*virF*Δ42N and pBl770::*virF*ΔATG<sub>(LP to AA)</sub> (Schrammeijer *et al.*, 2001) were digested with *SalI* and *EagI* and fragments inserted into *SalI*-NotI digested pAS2-1. This gave origin to
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The *GAL4BD* bait vectors pSDM3508, pSDM3509 and pSDM3510, respectively. A description of these and additional plasmids used in this study is given in Table 1.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
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<td>Clontech Laboratories, Inc. Palo Alto, CA</td>
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<td>GAL4-DNA binding domain bait vector, Cb, <em>LEU2</em></td>
<td>Kohalmi et al., 1998</td>
</tr>
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<td>pB1770::virF*AT</td>
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</tr>
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<td>pB1770::virF*AT</td>
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<td>Kohalmi et al., 1998</td>
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<td>Schrammeijer et al., 2001</td>
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<tr>
<td>pB1771::ASK2</td>
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<td>Schrammeijer et al., 2001</td>
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<td>pGEX-KG</td>
<td>Bacterial expression vector for GST tagged proteins, Cb</td>
<td>Guan and Dixon, 1991</td>
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<tr>
<td>pET16H</td>
<td>Bacterial expression vector for histidine tagged proteins, Cb</td>
<td>Novagen</td>
</tr>
<tr>
<td>pJS96</td>
<td>pYPGE2 (Brunelli and Pall, 1993) containing the <em>SKP1</em></td>
<td>Schouten, 1999</td>
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<td>pET16H::SKP1, Cb</td>
<td>This study</td>
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<tr>
<td>pSDM3214</td>
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<td>pET16H::Clone 44, Cb</td>
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<td>pSDM3528</td>
<td>pET16H::Clone 53, Cb</td>
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*a*Selectable auxotrophic markers for yeast two hybrid vectors: *TRP1* (phosphoribosylanthranilate isomerase in tryptophan biosynthesis), *LEU2* (β-isopropylmalate dehydrogenase in leucine biosynthesis). Cb: carbenicillin

**Yeast two-hybrid screening**

An *Arabidopsis thaliana* ecotype Columbia cDNA library was constructed from mRNA isolated from green tissue of six week-old flowering plants. cDNAs were cloned
transitionally as EcoRI-XhoI fragments to the GAL4AD sequence, encoding the Gal4-DNA Activation Domain, in the pACT2 prey vector (J. Memelink, Leiden University). The Matchmaker yeast two-hybrid system (Clontech Laboratories, Inc. Palo Alto, CA) was used to screen this library using the VirF-GAL4BD fusions (pSDM3508, pSDM3509 and pSDM3510) as a bait. Co-transformation of bait vectors and library was performed into the *Saccharomyces cerevisiae* strain PJ69-4A (MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL4-2ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ) (James et al., 1996) according to Gietz et al. (1995) using TRP1 and LEU2 as selectable markers. PJ69-4A contains two nutritional markers (HIS3 and ADE2) and an endogenous reporter gene (MEL1) driven by the GAL4-controlled GAL1, GAL2 and MEL1 promoters respectively (James et al., 1996). VirF-interacting clones were identified on their ability to grow in the absence of histidine (His) and adenine (Ade) at 20°C. To this end, we used as minimal medium Synthetic Dropout (SD) medium (SD: 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose, 1.5% bacto-agar) supplemented with uracil (Ura 0.02 g/l), methionine (Met 0.02 g/l), lysine HCL (Lys 0.03 g/l), histidine (His 0.02 g/l), leucine (Leu 0.03 g/l), tryptophan (Trp 0.02 g/l) and adenine (Ade 0.017 g/l), according to the requirement of the transformed strain, plus 1 mM 3-amino-1,2,4-triazole (3-AT). Then, individual His+ colonies obtained after 10 days in media lacking Leu, Trp and His (SD/-Leu/-Trp/-His) were sub-cultured on SD/-Leu/-Trp/-Ade to confirm adenine auxotrophy. In PJ69-4A the lack of adenine expression leads to the formation of red colonies, while wild-type cells form white colonies. Intermediate expression of adenine results in colonies in various shades of pink, where colony color could be used as an initial indication of the strength of an interaction (James et al., 1996). Ade+ colonies were then tested for X-α-Galactosidase activity conferred by GAL4 responsive MEL1 gene expression by growth of colonies that turned blue in basic SD medium lacking Leu and Trp but containing 20 μg/ml of the chromogenic substrate 5-Bromo-4chloro-3-indolyl-α-galactopyranoside (X-α-Gal, Clontech laboratories, Inc. Palo Alto, CA). Colonies that were able to grow in the absence of Leu, Trp, His and Ade, and metabolize the X-α-Galactosidase substrate (X-α-Gal) were regarded as putative interactors. For further analysis of cDNA clones identified by interaction with VirFΔ42N, the GAL4AD primers BC304 (5’-CTATTCGATGATGAAGATACC-3’) and IN069 (5’-TTGATTTGAGACTTGACC-3’) (Clontech Laboratories, Inc. Palo Alto, CA) were used to classify clones based on similar restriction enzyme patterns of PCR products, digested with the 4-base cutter enzymes Haelll and Rsal. In addition, pACT2 prey vectors for each clone (GAL4AD) were isolated and amplified in *E. coli* to determine the size of each interacting sequence by restriction with EcoRI and XhoI, and to attempt a possible classification by restriction with EcoRV and SalI. To confirm whether positively scored interacting colonies were not due to autoactivation, the isolated yeast cDNA clones were used to retransform the original pJ69-4A strain. Growth rates in absence of His and Leu of the original strain retransformed with the VirFΔ42N bait and the isolated cDNA clone prey vector (pJ69-4A + [GAL4BD-VirFΔ42N bait vector + prey GAL4AD-cDNA clone vector]) were compared with a set of controls: wild type pJ69-4A + prey GAL4AD-cDNA clone; pJ69-4A + [empty bait GAL4AD + prey GAL4AD-cDNA clone] and pJ69-4A + [empty bait GAL4AD + empty prey GAL4AD]). Selected clones were sequenced
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(Base Clear, The Netherlands) and BLAST analysis was performed at the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) and the Munich Information Center for Protein Sequences A. thaliana database (MATDB: http://mips.gsf.de/proj/thal/db/index.html).

Protein interaction using GST-pull down assays

To confirm the interactions detected by the yeast two-hybrid system, virF translational fusions to Glutathione S-transferase (GST) were constructed in the pGEX-KG vector (Guan and Dixon 1991). First, a fragment containing virF∆42N from pSDM3190 (Schrammeijer et al., 2003) was subcloned as XbaI-EcoRV (partial) in pIC19H (XbaI-SmaI) giving origin to pSDM3214. Then, virF∆ATG, virF∆ATG(ΔP to AA) and virF∆42N were cloned as SalI-HindIII fragments from pSDM3512, pSDM3513 and pSDM3214 in pGEX-KG, resulting in pSDM3514, pSDM3515 and pSDM3031, respectively. The cDNA sequences of the interacting yeast two-hybrid clones were obtained by PCR using the A. thaliana pACT2 cDNA library as template. For amplification of the corresponding full length DNA sequence of clones 22, 33 and 54, which lack intron sequences, genomic DNA of A. thaliana ecotype Columbia was used. Primers, which were designed based on the gene sequence of the two-hybrid clones and genome BLAST searches, are described in Table 2. The coding sequences were cloned in the expression vector pET16H (Novagen), resulting in a His-tag at the N-terminus of the expressed proteins (pSDM3516 to pSDM3518 and pSDM3520 to 3528).

<table>
<thead>
<tr>
<th>cDNA clone</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>5</td>
<td>5'-GGATTCTCATTATGGAATTCTCTCCTTTACTGCAAGAATCTCCCTTCT-3'</td>
<td>5'-CCGAATTCTTACTGCAAGAACTCCCTTGG-3'</td>
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Table 2. Primers used for amplification of cDNA clones

66
The GST fusion proteins were expressed in *Escherichia coli* strain BL21 (Novagen). To this end, 25 ml of LC medium (10 g/L bacto-tryptone, 5 g/L bacto-yeast extract and 8 g/L NaCl, pH 7.0) containing chloramphenicol (50 mg/L) and carbenicillin (100mg/L) was inoculated to a final OD<sub>600</sub> of 0.2 with cells from an overnight culture and incubated at 37°C during 4 hours (OD<sub>600</sub> ≈ 0.5 to 0.7). Induction with 1 mM IPTG was done overnight at 18°C or for 3 hours at 30°C. Cells were collected in aliquots of 4 ml (4000 rpm 5 min) and resuspended in 1 ml of extraction buffer (150 mM NaCl, 2 mM KCl, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM EDTA, 2 mM DTT, 0.1% Triton X-100, pH 7.6 and protease inhibitors as phenylmethylsulfonyl fluoride (PMSF) (100 μg/ml), antipain (1.0 μg/ml) and leupeptin (1.0 μg/ml).

After sonication for 2 minutes, cell lysates in extraction buffer were centrifuged at maximum speed for 15 minutes. Supernatants were added to Glutathione-Sepharose matrix (Amersham-Pharmacia) and rocked for 1-2 hours at 4 °C. To study *in vitro* interaction of the His-tagged *Arabidopsis* proteins with GST::VirF, cells expressing the His-tagged proteins were similarly treated and his-tagged extracted proteins were incubated with the GST::VirF bound Glutathione-matrix during 4 hours at 4°C in binding buffer (50 mM Tris-HCL, pH 6.8; 100 mM NaCl; 10 mM CaCl<sub>2</sub>; 0.1% Triton X-100 and protease inhibitors as described in extraction buffer). After several washes in binding buffer (with centrifugation for 1000 g/5 min per wash) the beads were resuspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and proteins were separated in a 12.5% polyacrylamide gel. Proteins were blotted to a polyvinylidine fluoride (PVDF)-Immobilon P-Millipore membrane using a semidry blotting apparatus (2117 Multiphor II electrophoresis unit from LKB [Upsala]), and blotting buffer (39 mM glycine, 48 mM Tris- HCl, 0.0375% SDS, 20% Methanol). Detection of His-tagged interactors was performed by Western blot analysis using penta-His antibodies (1:2000 dilution) (Qiagen, Benelux B.V.).

**Yeast PCR and Dot Blot assays**

Yeast two-hybrid cultures expressing the full-length cDNA clones that interacted with VirF were grown overnight in 10 ml of SD medium lacking Trp and Leu. Two ml aliquots of cell culture were pelleted (13000 rpm/30 sec) and resuspended in 200 μl of 0.9 M sorbitol/50 mM EDTA (pH 8.0) containing 4 mg/ml of lyticase (*Arthrobacter luteus*, 750 U/mg, Sigma). To obtain spheroplasts the mixture was incubated at 30°C for 60 min and centrifuged at 3000 rpm for 5 min. Plasmid isolation from the pellet was performed as reported by Birnboim and Doly (1979) or standard Qiagen plasmid miniprep columns. The obtained DNA was directly used in the following experiments.

In order to determine the presence of ASK1 among the interactors of VirF, a PCR screening to obtain the full length ASK1 coding sequence was performed using the ASK1-C-ter reverse primer (5'-AATCATTCAAAAGCCCATTTG-3') in combination with the pACT2 GAL4AD forward primer (BC304) (Clontech Laboratories, Inc. Palo Alto, CA). The cDNA plasmids of the yeast two-hybrid clones identified in the screen with full-length VirF were used as template for PCR. Besides, amplification with the pACT2 internal primers BC304 and IN069 (Clontech Laboratories, Inc. Palo Alto, CA) was performed for all the clones to
verify the presence of a cDNA clone in the selected pACT2 library plasmids. pBI771::ASK1 and pBI771::ASK2 (Schrammeijer et al., 2001) were used as positive controls and pBI771-GAL4AD vector and wild type strain DNA were used as negative controls. Reaction conditions using Red-Taq polymerase (Sigma) were 95°C for 1 min, 35 cycles of 1 min 95°C, 1 min 46°C and 2 min at 72°C with a final extension of 10 min at 72°C.

For dot blot analysis, a 15x dilution in denaturing solution (0.5 M NaOH, 1.5 M NaCl) of each positive PCR reaction for the primers BC304 and IN069 (Clontech Laboratories, Inc. Palo Alto, CA) was prepared and 2 µl was spotted on a positively charged nucleic acid transfer Hybond-N+ membrane (Amersham). Plasmid DNA of pBI771::ASK1, pBI771::ASK2 and PCR products of pBI771::ASK1 and pBI771::ASK2 were spotted as positive controls. To exclude cross hybridization with the SKP1 gene of S. cerevisiae, the cloned gene was used in the dot-blot assay as control. SKP1 was obtained by PCR using primers SKP1-1 (5”-CCC TCGAGGGTGACTTCTAATGTGGTCC-3”) and SKP1-2 (5”-GGGGTACCCTAACGGTCTTC AGCCC-3”) using as template pJS96 (Schouten, 1999). The fragment was cloned after digestion with XhoI and KpnI in pET16H resulting in pSDM3034. DNA of pSDM3034 and the ‘empty’ pACT2 vector were used as negative controls. A non-radioactive probe (Digoxigenin labeling) containing the full-length ASK1 gene was obtained using primers ASK1 forward (5”-ATGTCTGCGAAGAAGATTGTGT-3”) and ASK1-C-ter reverse (5”-AATCATTCAAAAGCCCCATTG-3”) on pBI771::ASK1 (Schrammeijer et al., 2001) according to the manufacturer’s conditions. Hybridization conditions were according to Boehringer Mannheim kit. Detection of DNA hybrids by chemiluminescence method was performed with the substrate CDP-Star™ (Roche Diagnostics GmbH, Mannheim, Germany). All test procedures and buffer preparations were carried out according to the instructions of the manufacturer.

RESULTS

Isolation of putative target proteins of VirF

Previously, two Arabidopsis SKP1-like proteins, ASK1 and ASK2, were identified as interactors of VirF in a yeast two hybrid analysis. The interaction with ASK1 and ASK2 proteins was mediated by an N-terminal F-box domain and indicated that VirF might function as a part of the protein degradation machinery inside the plant cell (Schrammeijer et al., 2001). To identify putative target proteins of VirF, we used a VirF protein lacking this F-box domain (VirFΔ42N) and a VirF mutant in the functional F-box motif (VirFΔATG_(LP to AA)) as baits in a yeast two-hybrid screen. We anticipated that these mutant proteins would allow to specifically identify target proteins of VirF and not ASK proteins. However, we also included full length VirF as bait in case incorrect folding of the other versions would lead to decreased binding affinity with target proteins. In contrast to the system used by Schrammeijer et al. (2001), we used the Matchmaker yeast two-hybrid system (Clontech Laboratories, Inc. Palo Alto, CA) based on the pAS2.1 and pACT2 bait and prey vectors, which are high copy replication units. The three different versions of virF were fused translationally to the GAL4-DNA Binding Domain (GAL4BD) in pAS2.1. The yeast strain PJ69-4A contains adenine, histidine and X-α-galactosidase reporter genes, which become activated by Gal4-mediated transcriptional activation of the GAL1, GAL2 and MEL1 promoters respectively (James et al.,
1996). The Gal4BD-VirF fusion proteins did not show auto-activation of these reporter genes. To identify interacting Arabidopsis proteins, we independently screened an A. thaliana cDNA library constructed in a GAL4DNA Activation Domain (GAL4AD) vector (pACT) with the three different versions of VirF. The transformation efficiency, the total number of transformants obtained and the number of positive interacting clones after a first selection based on histidine, adenine and X-α-galactosidase expression are summarized in Table 3. The putative interacting clones were re-plated on selection medium lacking histidine. This resulted in a reduction of false positives by approximately 30%, finally resulting in 56 putative interacting clones with VirFΔ42N, 110 clones with VirFΔATG,(LP to AA) and 171 clones with VirFΔATG.

Table 3. Transformation details of an A. thaliana cDNA library screening with different VirF baits

<table>
<thead>
<tr>
<th>GAL4BD-VirF fusion</th>
<th>Transformation efficiency (colonies/µg DNA)</th>
<th>Total number of transformants</th>
<th>Colonies His+, Ade+, X-αGal +</th>
<th>Colonies after re-plating on SD His-</th>
<th>Colonies after retransformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>VirFΔ42N</td>
<td>2 x 10⁴</td>
<td>8 x 10⁵</td>
<td>69</td>
<td>56</td>
<td>21</td>
</tr>
<tr>
<td>VirFΔATG,(LP to AA)</td>
<td>4x 10³</td>
<td>2 x 10⁵</td>
<td>112</td>
<td>110</td>
<td>ND</td>
</tr>
<tr>
<td>VirFΔATG</td>
<td>7 x 10³</td>
<td>3 x 10⁵</td>
<td>330</td>
<td>171</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: Not determined

To investigate which of the selected clones were potential targets for SCF<sup>VirF</sup>-complex-mediated protein degradation by the 26S proteasome during the infection process, we continued the analysis of the 56 clones that showed interaction with VirFΔ42N. In order to classify the clones previous to yeast retransformation, we performed restriction analysis on PCR products amplified from DNA isolated from yeast clones containing the interacting clones with the 4-base cutters enzymes HaeIII and RsaI. The DNA gel patterns obtained with each enzyme resulted in a different pattern for practically each clone (data not shown). In addition, we determined the size of the cDNAs, after re-transformation and isolation of DNA from E. coli, by restriction with EcoRI and XhoI, which were used to construct the Arabidopsis cDNA library. The restriction patterns showed fragments with sizes varying between 0.2 to 2.5 kb (Figure 1), some of the clones gave fragments of the same size. However, after a second restriction using EcoRV and SalI, no common bands apart from an expected 800 bp form the vector itself could be observed. The large variation between the clones based on PCR and restriction data made it impossible to classify the cDNA clones, and indicated that we had picked up many different interactors, or had problems with high background levels.
Isolation of *Arabidopsis* protein interactors of VirF

**Figure 1.** *EcoR*I–*Xho*I digestion of pACT-cDNA prey plasmids from the VirFΔFbox (*VirFΔ42N*) screening showed one, two or three DNA fragments (0.2 to 2.5 kb) for different clones. Some of these are depicted here: **A**) clones 17 to 49. **B**) Clones 15 and 50 to 57. Clones 1 to 14, 16, 18, 19, 20, 21, 24, 27, 29 and 30 are not shown. Several of the clones displayed the same single band restriction pattern, as is the case for clones 17, 22, 23, 32 and 56 (550 bp); 29, 41, 42 and 57 (950 bp); 18, 24 and 47 (1200 bp); 10, 13 and 55 (1250 bp); 31, 33 and 34 (1350 bp); 20 and 38 (600 bp) 15 and 21 (700 bp); 9 and 46 (800); 44 and 53 (900 bp) and 27 and 28 (1100 bp). Two bands were found for clones 25 and 26 (200 and 450 bp) and even three bands for clones 45 and 49 (300, 450 and 2300 bp). The rest of the putative interacting clones had all different patterns with one (3, 5, 14, 50, 52 and 54) two (1, 2, 4, 8, 12, 16, 30, 35, 36, 40, 43 and 48) or three bands (39). Restricted empty GAL4 activation domain prey plasmid (pACT) and unrestricted clone 22 (22*) were used as controls. M: EUROGENTEC DNA ladder.

Consequentially, the GAL4AD-cDNA plasmids were isolated and retransformed to PJ69-4A yeast cells containing an empty bait vector (*GAL4BD*) or the *GAL4BD::virFΔ42N* plasmid. Transformation of 20 out of the initial 56 cDNA clones resulted in growth of transformants on selection medium lacking His or Ade and showed X-α-galactosidase activity when the plasmid *GAL4BD::virFΔ42N* was also present, but not when the cDNA clones were introduced in empty yeast and yeast containing an empty bait vector. This indicated that these 20 clones did not auto-activate the reporter constructs (data not shown). Four additional clones grew well in the absence of His but showed no clear growth on media lacking Ade. Re-plating His+ colonies to SD medium minus Ade identified only one of these clones (number 8) as an additional possible interactor of VirF, resulting in a total of 21 putative interacting clones after the second yeast two-hybrid round. Five of the stronger interactions are shown in figure 2.
Figure 2. Yeast two-hybrid analysis evidenced protein interaction of VirF\textDelta Fbox (VirF\textDelta 42N) after introduction of prey cDNA plasmid in bait containing yeast. Cells were plated out in absence of histidine (SD-Try-Leu-His) or adenine (SD-Try-Leu-Ade) and His\textsuperscript{+} and Ade\textsuperscript{+} colonies were tested for X-\textalpha-galactosidase activity. Growth was compared to autoactivation control in which prey plasmid was introduced in empty yeast. C(+): positive control yeast containing bait (VirF) and prey (ASK1) vectors; C(-): negative control yeast containing empty bait and prey vectors.

Presence of ASK1 homology among full-length interactors
We were surprised by the high number of putative interacting A. thaliana clones here identified using each of the three BD-VirF fusions as bait in the yeast two-hybrid screens (see Table 2), as Schrammeijer et al., (2001) described the identification of only two plant proteins as interactors of VirF, ASK1 (18 times), and ASK2 (once), after using full-length VirF as a bait. Therefore, we analyzed whether the high number of cDNA clones identified in our screen with full length VirF (171) also mainly represented ASK homologues. To identify the presence of ASK1 among the selected interacting clones, we performed a PCR screening using a combination of primer BC304 (annealing in the pACT2 plasmid) and primer ASK1-\textsuperscript{C-ter}. As an internal control we used pACT2 primers BC304 and IN069 (Clontech Laboratories, Inc. Palo Alto, CA), which will amplify any cloned cDNA. Amplification with the positive control was obtained in 137 out of 171 cDNA clones (80\%). Of these 137 clones, however, only three (2.2\%) were positive for ASK1. To confirm the PCR data we performed a DNA dot blot assay. To this end, DNA fragments from each of the clones amplified with the primers BC304/IN069 were spotted on a membrane. ASK1 DNA was detected using a digoxigenin labeled ASK1 full-length probe (483 nucleotides). We did not detect cross hybridization with the yeast SKP1 gene. In addition, although ASK1 and ASK2 show 69.5\% homology, we were not able to detect a positive signal of ASK2 control DNA (data not shown). Therefore, we
could only identify *ASK1* clones, but no other genes of the *ASK* family (Reviewed in Moon, Parry and Estelle, 2004). DNA from only two of the three cDNA clones that were positive for *ASK1* with PCR showed a hybridization signal in the dot blot assay (data not shown). Summarizing, the data suggest that in contrast to the results obtained by Schrammeijer *et al.* (2001) in this yeast two hybrid screen we selected many different cDNA clones using both full length VirF as well as the VirFΔ42N fusion as bait. This may either point to a high background due to the high copy number of the plasmids pAS2.1 and pACT2, or suggest an efficient two-hybrid system with which also weakly interacting clones can be detected. Subsequent *in vitro* and *in vivo* studies are therefore crucial to determine whether the identified proteins are true targets of VirF inside the host plant during the infection process.

**Figure 3. Protein expression of translational fusions in E. coli.** A) Visualization by Coomassie Blue staining of N-terminal Glutation S-transferase fusions to full-length sequence and deleted versions of VirF: GST::VirF (48 kDa), GST::VirFΔLP (48 kDa), GST::VirFΔbox (43 kDa) and GST (26 kDa). Samples are loaded in duplicate. From left to right: second last line was unloaded; last lane contained Invitrogen Mark 12 Standard. Black arrows indicate the expected bands. B) Western blot analysis with histidine antibodies to detect His-tagged VirF-interacting clones. Seven proteins were expressed: Lon protease-like (clone 15, 31.3 kDa), hypothetical catalase protein (clone 16, 54.2 kDa), glutamate-ammonia ligase precursor (clone 18, 47.3 kDa), 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase 2-DHS2 (clone 47, 56.1 kDa), ATPase B-subunit-VHA-B3 (clone 40, 52 kDa), pirin-like (clone 39, 32.6 kDa) and branched-chain amino-acid aminotransferase (clone 44, 39 kDa). Clones 5, 8, 53, 55 and the three homologues for the putative jasmonic acid inducible protein or EPS protein (clones 22, 33, 54) were not expressed. C (-): negative control for expression pET16H empty vector. For identification of unexpressed proteins the reader is referred to Table 4. Detection of His-tagged interactors was performed by Western blot analysis using penta-His antibodies (1:2000 dilution) (Qiagen, Benelux B.V.).
Description of the isolated VirF interacting proteins

The DNA sequence of the 21 putative interactors pulled out with GAL4BD::virFΔ42N was determined, and BLAST analysis was performed using DNA and protein sequence databases. As expected from previous PCR analyses almost all of these 21 DNA sequences matched a different entry in the Arabidopsis thaliana MATDB (http://mips.gsf.de/proj/thal/db/Index.html) and GenBank (http://ncbi.nlm.nih.gov/BLAST/) databases (summarized in Table 4), suggesting that VirF may have multiple interactors. A gene encoding a Lon-protease like protein (gene number AT1G75460) was represented twice (clones 15 and 17) and a gene encoding epithiospecifier protein ESP (gene number AT1G54040) was selected three times (clones 22, 33 and 54). The remaining 16 putative interactors were identified only once (Table 4). As can be seen in Table 4, several of the 16 interacting proteins are involved in metabolic processes or defense and stress related processes. It is remarkable that some of the selected proteins (including DHS2, aconitase, Lon-protease like, GS2) carry putative chloroplast or mitochondrial localization signals.

In vitro interaction of VirF with host proteins using GST pull-down assays

To confirm the interactions detected by the yeast two-hybrid screening using GST pull down assays, we selected the two cDNAs that were identified more than once (clones 15/17 and clones 22/33/54), as well as 10 other cDNA clones (5, 8, 16, 18, 39, 40, 44, 47, 53 and 55). Comparisons with the annotated genome sequence had shown that most selected plasmids contained partial cDNA clones. Primers were therefore designed to amplify the full coding sequence of each of the genes (Table 2) from an A. thaliana cDNA library. Most cDNAs were amplified successfully. Unfortunately, we were unable to successfully amplify the complete cDNA sequence encoding the epithiospecifier protein (ESP, AAL14623.1; 341 AA) that we identified three times in our screen. Two different annotations of the ESP gene exist. The larger version has a predicted intron sequence, but an intronless annotated version is also described, which encodes a 261 AA putative jasmonic acid inducible protein (JAIP, AAL69516.1), that lacks the N-terminal 80 AA of the annotated ESP. The cDNA clones 22, 33, and 54 all represent partial cDNA sequences of the ESP gene. Whereas both clones 33 and 54 start at the same position, similar to the start of the annotated JAIP coding sequence, clone 22 encodes only the C-terminal 49 AA of ESP. Our initial attempts to amplify the ESP cDNA sequence may have failed due to absence of the complete ESP cDNA sequence in our library. Therefore, we designed new primers and successfully amplified a smaller portion of the gene sequence encoding the C-terminal 49 AA of ESP, which was originally identified in our yeast 2-hybrid screen (clone 22) to interact with VirF, and should therefore contain a VirF-interaction domain.

We cloned the PCR products of the selected clones in pET16H, which upon expression results in proteins with a His-tag at the N-terminus. The different versions of the virF gene were translationally fused to the glutathione-S-transferase (GST) gene resulting in GST::virFΔ42N, GST::virFΔATG LP to AA and GST::virFΔATG. The GST and His-fusion plasmids were expressed in E. coli BL21-DE3 cells. Expression of the GST-VirF fusions after IPTG induction was analyzed by GST pull-down of total cell extract using Glutathione-
Isolation of *Arabidopsis* protein interactors of VirF

Sepharose beads followed by PAGE and Coomassie blue staining (Figure 3A). Seven of the 12 cDNAs (derived from clones 15, 16, 18, 39, 40, 44 and 47) showed expression in *E. coli* as shown by Western blot analysis using His antibodies for detection of the His-fusion proteins (Figure 3B). All expressed proteins were of the expected size (see legend to Figure 3). Although growth of the other five bacterial cultures was not affected, we were unable to detect expression of the cDNAs (including partial cDNA clone 22) under the conditions described in the experimental procedures section.

![Diagram A](image)

**Figure 4. VirF interaction with plant proteins.** A) Western blot analysis showed *in vitro* binding by pull down assays using extracts of induced cells containing GST::VirFΔ42N (+) and His-tagged plant interacting clones in pET16H (PIF): Lon protease-like (31.3 kDa), pirin-like (32.6 kDa), branched-chain amino-acid aminotransferase (39 kDa), ATPase B-subunit VHA-B3 (52 kDa) and predicted 2-deoxyphosphoheptonate aldolase/3-deoxy-7-phosphoheptulonate 7-phosphate (DAHP) synthase 2 (DHS2) (56.1 kDa). Induced cells containing GST empty pGEX-KG vector were used as negative control for binding (-). B) *in vitro* binding of Lon protease-like protein (31.3 kDa) to the three different GST translational fusions of VirF (+). Here, empty pET16H (histidine tagging vector) was used as negative control for binding to GST::VirF fusions (-). Note that a weaker band appears when testing binding to GST lacking VirF (empty pGEX-KG vector) that due to longer exposure shows as background. Detection of His-tagged interactors was performed by Western blot analysis using penta-His antibodies (1:2000 dilution) (Qiagen, Benelux B.V.).

![Diagram B](image)
Next, we performed a pull-down assay using GST-VirFΔ42N-coated glutathione beads followed by Western blot analysis, and showed that four of the seven expressed His-protein fusions (15 [Lon-protease like], 39 [a putative pirin like protein], 40 [vacuolar H+ ATPase subunit B3-VHA-B3] and 47 [3-deoxy-7-phosphoheptulonate 7-phosphate (DAHP) synthase 2-DHS2]) showed interaction with GST-VirFΔ42N, whereas no binding was observed with GST alone (Figure 4A). Although clone 44 showed a low efficiency of binding to GST alone, a higher intensity of the signal was obtained after pull down with GST::VirFΔ42N, and made us decide to include this branched-chain amino acid transferase for further analysis. To have and idea whether the binding properties to the PIF proteins were maintained with longer versions of VirF, we also confirmed binding of one clone, the Lon-protease, to GST-VirFΔATG (LP to AA) and GST-VirFΔATG (Figure 4B). After this, our work was only based on binding assays to GST-VirFΔ42N and taken as data for VirF.

The data show that five of the seven clones that were expressed in E. coli interacted with VirF not only in yeast 2 hybrid (Figure 2), but also in in vitro GST pull down assays. Clones 16 and 18, although expressed in E. coli, did not bind to VirF in vitro, suggesting that catalase 3 and glutamine synthetase 2, respectively, are not targets of VirF during infection. However, incorrect post translational processing or folding of the eukaryotic proteins in E. coli may also have caused the inability to interact in vitro.

### Table 4. VirFΔF-box Interacting proteins from *A. thaliana* cDNA library screening

<table>
<thead>
<tr>
<th>cDNA clone</th>
<th><em>A. thaliana</em> gene accession number/ Gene Bank entry</th>
<th>Times found</th>
<th>Encoded protein sequence (Gene Bank)</th>
<th>Main properties reported in plant and eukaryotic related proteins</th>
<th>Related references</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 At4g13430 (NM_117417)</td>
<td>1</td>
<td>Aconitase (NP_567405.1)</td>
<td>- Leucine biosynthesis. - Domain: Pfam profile PF00330: Aconitase family (aconitate hydratase-CDD:30079); Glycine rich ATP/GTP binding site motif A (P loop).</td>
<td>Gruer, Artymiuk and Guest, 1997 Town et al., 2002.</td>
<td></td>
</tr>
<tr>
<td>8 At4g01620 (AK176330)</td>
<td>1</td>
<td>Cathepsin β-like cysteine protease (BAD44093.1)</td>
<td>- Thiol (cysteine) protease active site (cysteine- type endopetidase activity). - Domain: Peptidase_C1A_CathepsinB (CDD:30294).</td>
<td>Dufour, 1988; UniprotKB/TrEMBL entry Q9ZSI0.</td>
<td></td>
</tr>
<tr>
<td>15 (17) At1g75460 (AY045939)</td>
<td>2</td>
<td>Lon protease-like (AAK76613 )</td>
<td>- Domain: N-terminal ATP-dependent protease La (Lon) domain, (Pfam PF02190) (ATP-dependent serine peptidases from the MEROPS peptidase family.</td>
<td>Barakat et al., 1998Ebel et al., 1999 Chandu et al., 2004 Rawlings et al., 2006.</td>
<td></td>
</tr>
<tr>
<td>16 At1g20620 (AY058104)</td>
<td>1</td>
<td>Catalase 3 (SEN2) (AAL24212;)</td>
<td>- Hydrogen peroxide catabolic processes. - Expressed during plant senescence in inflorescence and leaf and plant cell rescue, defense and detoxification related processes. - Domain: Catalase: IPR002226.</td>
<td>Zhong and McClung, 1996; Park et al., 1998; Heazlewood et al., 2004.</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Continuation

<table>
<thead>
<tr>
<th>cDNA clone</th>
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<th>Related references</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 At5g35630 (AY122977)</td>
<td>1</td>
<td>Glutamine synthetase2 GS2 (AAM67510.1)</td>
<td>- Glutamine biosynthesis. - Domains: Pfam00120 (Gln-synt_C: Glutamine synthetase, catalytic domain-CDD:40220); Pfam 03951 (Gln-synt_N: Glutamine synthetase, beta-Grasp domain-CDD:43868).</td>
<td></td>
<td>Peterman and Goodman, 1991.</td>
</tr>
<tr>
<td>20 At3g54470 (X71842)</td>
<td>1</td>
<td>Orotate phosphoribosyl transferase/ 7Orotidine 5'-phosphate decarboxylase: I54/ PyrE-F (CAA50866)</td>
<td>- Pyrimidine biosynthesis. - Domains: Orotidine 5'-phosphate decarboxylase: IPR001754, Orotate phosphoribosyl transferase: IPR004467, Purine/pyrimidine phosphoribosyl transferase: IPR002375, Phosphoribosyltransferase: IPR000836.</td>
<td></td>
<td>Nasr et al., 1994; Schröder, Giermann and Zrenner, 2005.</td>
</tr>
<tr>
<td>22 At1g54040 (AY074550) (33) At2g43120 (NM_180054) (54) AF416787</td>
<td>3</td>
<td>Epithiospecifier protein-ESP (AAL14623.1)</td>
<td>- Response to jasmonic acid stimulus. - Non-catalytic cofactor in the hydrolysis of glucosinolates to epithionitriles by myrosinase. The products of this hydrolysis have been correlated with plant defense. - Domains: Kelch repeat: IPR006652 (4 times) Pfam01344 (CDD:41396).</td>
<td></td>
<td>Lambrix et al., 2001; Matusheski et al., 2004; Zabala et al., 2005; Burow et al., 2006.</td>
</tr>
<tr>
<td>39 At2g43120 (NM_180054)</td>
<td>1</td>
<td>A. thaliana pirin-like protein (NP_950385)</td>
<td>- Apoptosis of human cells and plant defense responses. - Domains: Pirin (Pfam PF02578; CDD:42639) member of the Cupin family; calmodulin binding domain.</td>
<td></td>
<td>Hoeberichts et al., 2001; Orzaez et al., 2001.</td>
</tr>
<tr>
<td>40 At1g20260 (NM_101877)</td>
<td>1</td>
<td>Vacuolar H+ ATPase subunit B3 (VHA-B3)/A. thaliana ATP binding/hydrogen -transporting ATP synthase, (NP_173451.2)</td>
<td>- ATP synthesis coupled proton transport, cytosolic pH regulation. - The V-type (in vacuoles and clathrin-coated vesicles) pump H+. - Domains: Pfam profiles PF00006: ATP synthase alpha/beta family nucleotide-binding domain; PF02874: ATP synthase alpha/beta family beta-barrel domain.</td>
<td></td>
<td>Stevens and Forgac, 1997; Seol et al., 2001; Kluge et al., 2003; Martiniova, Maeshima and Neuhaus, 2007.</td>
</tr>
<tr>
<td>43 At2g26080 (NM_128167)</td>
<td>1</td>
<td>A. thaliana glycine dehydrogenase (decarboxylating) (NP_180178.1)</td>
<td>- Glycine decarboxylation via glycine cleavage system. - Domains: Glycine cleavage system P-protein: IPR03437.</td>
<td></td>
<td>Town and White, 2002; Heazlewood et al., 2003.</td>
</tr>
<tr>
<td>44 At3g19710 (NM_112861)</td>
<td>1</td>
<td>Branched-chain aminotransferase BCAAT4 (NP_188805.1)</td>
<td>- Involved in methionine derived glucosinolates biosynthesis. - Domains: Pfam profile: PF01063 aminotransferase class IV.</td>
<td></td>
<td>Schuster, 2006.</td>
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<tr>
<td>47 At4g33510 (NM_119505)</td>
<td>1</td>
<td>DHS2 (3-deoxy-7-phosphohexulonate (DAHP synthase 2) (NP_195077.1)</td>
<td>- Aromatic amino acid biosynthesis (Shikimate pathway). - Class-II DAHP synthetase family; Pfam 0147 (CDD:41520). - Domains: DAHP synthetase, classII:IPR02480(2) DHS1: Induction by wounding and pathogenic attack</td>
<td></td>
<td>Keith et al., 1991; Gorlach et al., 1993; Jones et al., 1995; Entus et al., 2002.</td>
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</tbody>
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## Table 4. Continuation

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<tr>
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<th>Main properties reported in plant and eukaryotic related proteins</th>
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</thead>
<tbody>
<tr>
<td>48 At2g23420 (NM_127906)</td>
<td>1 Nicotinate phosphoribosyl transferase family protein / NAPRTase family protein (NP_179923)</td>
<td>- NAD biosynthesis, nicotinate nucleotide biosynthesis. - Domain: Nicotinate phosphoribosyltransferase (NAPRTase) family: PF04095.</td>
<td>Town et al., 2003.</td>
<td></td>
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<td>53 At1g56070 (AY054461.1)</td>
<td>1 Elongation factor EF-2 (AAK96653.1)</td>
<td>- Low similarity to BRE alpha-b isoform (Homo sapiens). - Domains: Brain and reproductive organ-expressed protein (BRE): Pfam 06113.</td>
<td>Patel et al., 2002; Guo et al., 2002.</td>
<td></td>
<td></td>
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<tr>
<td>55 At5g42470 (NM_123812)</td>
<td>1 A. thaliana unknown protein (NP_190662.2)</td>
<td>- Aromatic compound metabolism, electron transport, removal of superoxide radicals. - Domains: FAD-dependent pyridine nucleotide-disulphide oxidoreductase:IPR001327; Adrenodoxin reductase:IPR000759; NAD-binding site:IPR000205; Flavin-containing monooxygenase FMO:IPR000960.</td>
<td>Town et al., 2003.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56 At1g67090 (AY0610101)</td>
<td>1 Ribulose bisphosphate carboxylase-like protein, small subunit (AAL38277.1)</td>
<td>- Carbon fixation.</td>
<td>Kawamura and Uemura, 2003.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>57 At1g12140 (NM_101086)</td>
<td>1</td>
<td>- Aromatic compound metabolism, electron transport, removal of superoxide radicals. - Domains: FAD-dependent pyridine nucleotide-disulphide oxidoreductase:IPR001327; Adrenodoxin reductase:IPR000759; NAD-binding site:IPR000205; Flavin-containing monooxygenase FMO:IPR000960.</td>
<td>Town et al., 2003.</td>
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Concluding, we confirmed in vitro interaction with VirF for five putative interactors isolated in the yeast 2H screening (Figure 2). We propose to name these “Plant Interactors of VirF” (PIF): PIF1 (3-deoxy-D-arabino-heptulosonate 7-phosphate [DAHP] synthase 2 [DHS2], an aldolase, clone 47), PIF2 (Lon protease like, clones 15 and 17), PIF3 (pirin like protein, clone 39), PIF4 (vacuolar H+ATPase subunit B [VHA-B3], clone 40) and PIF5 (branched-chain amino-transferase BCAT4, clone 44).

### DISCUSSION

The A. tumefaciens VirF protein is directly translocated from bacteria to host cells (Vergunst et al., 2000; Schrammeijer et al., 2003). The presence of an F-box domain allows interaction with the ASK1 and ASK2 proteins (Schrammeijer et al., 2001). The ASK proteins
play an important role in the function of E3-SCF Ub ligases by their binding to F-box proteins that recognize specific targets for degradation via the 26S proteosome. It is thus possible that VirF mediates the degradation of specific proteins in the host cell. To obtain more information about the role of VirF inside host cells, we approached the identification of plant proteins interacting with VirF by a yeast two-hybrid strategy. Interestingly, we found a large diversity of putative interacting partners of VirF.

**The screening procedure**

In a previous yeast two-hybrid screening with full length VirF as a bait, ASK1 and ASK2 proteins were obtained as interactors (Schrammeijer et al., 2001). We anticipated that it would thus not be possible to identify other interactors than the ASK proteins using the full length protein as a bait. Therefore, in the current study we used VirFΔ42N, which lacks the F-box to which ASK proteins bind. Using VirFΔ42N as a bait we eventually obtained from 8x10^5 transformants 21 clones, representing 18 different genes, encoding proteins interacting with VirF outside the F-box. The diversity of the encoded proteins by these 18 genes was surprising. This could be due to the fact that also weak interactors had been picked up or to an abnormal conformation of the VirF protein partly due to the absence of the F-box.

In order to obtain some insight in this, we analyzed whether the interactors obtained with the full length VirF protein as a bait were represented by ASK proteins as this was the case in the screening by Schrammeijer et al. (2001). To this end we analyzed the clones by PCR and dot blot analysis. The results showed that only a minority of the clones encoded the ASK1 gene. It is thus most likely that the screening performed had been less stringent than that done by Schrammeijer and collaborators and that for this reason, more weakly interacting proteins have been identified. Also it has to be mentioned here that the cDNA library was made from mRNA obtained from the green parts of six week-old A. thaliana seedlings, whereas the library used by Schrammeijer et al. was made from RNA obtained from a pool of two weeks-old seedlings mixed with mature flowering plants. These libraries may therefore differ in the diversity and the representation of particular mRNAs.

Whereas the screening by Schrammeijer was done with low copy vectors, the current screening employed high copy vectors. This may especially have facilitated the detection of interactors of varying strength. Also the yeast strain used, PJ69-4A (James et al., 1996), allows the efficient detection of interactors in contrast to some other yeast strains (Abbink et al., 2002). This is partially due to the fact that lower levels of 3-AT are employed to suppress background growth in the absence of histidine. The presence of higher concentrations of 3-AT may hinder the detection of weak interactors.

Out of the 18 different putative interactors we have identified in this screen, 12 were chosen for further analysis in GST pull downs. However, only seven clones were expressed at detectable levels in *E coli* using the buffer conditions described. Of these seven, Catalase 3 and GS2 did not interact with VirF in a GST pull down. Five of the identified proteins represent direct interactors of VirF (PIF) as established in pull down assays. It is for the future to determine whether the proteins truly interact *in vivo* in the plant host cells.
VirF Interactors

During our studies Tzfira et al., (2004) published that the bZip protein VIP1 was degraded in the presence of VirF. Indirectly, they found, this also led to the degradation of VirE2, the protein coating the T-strand, and a model was suggested that VirF is involved in uncoating of the T-strand, prior to T-DNA integration in the genome. The VIP1 protein was not identified in our current screens, Tzfira et al. (2004) indicated that VirF co-localized with VIP1 in the host cell nucleus. As VirF does not have a nuclear localization signal (NLS), VirF may enter the nucleus in a complex with VIP1 and mediate its proteolysis in the nucleus. However, our immunolocalization studies (Chapter 6) reveal the presence of VirF in the cytosol, suggesting additional mechanisms of action for VirF than uncoating the T-strand. Several of the interactors identified in our screen probably have organellar localization (chloroplast, mitochondrion). Binding may occur in the cytosol immediately after synthesis, and proteolysis may follow either after transfer to the organelle (nucleus) or in the cytosol as the chloroplast and mitochondrion are lacking the proteasome. It is not without precedent that effector proteins interact with the chloroplast or chloroplast proteins. For instance, recently it was found that the Tobacco mosaic virus (TMV) p50 effector mobilizes the protein NRIP1, which normally localizes to chloroplasts, to the cytoplasm and the nucleus (Caplan et al., 2008). It was mentioned that p50 may directly mask the chloroplast transit peptide or indirectly disrupt the chloroplast protein import machinery.

Several of the interactors identified (Table 4) are involved in metabolic processes. This is the case for clone 5 encoding aconitase, which is involved in leucine biosynthesis; clone 20 encoding orotate phosphoribosyl transferase/orotidine 5 phosphate decarboxylase, which is involved in pyrimidine biosynthesis; clone 43 encoding glycine dehydrogenase involved in glycine decarboxylation; clone 47 encoding 3-deoxy-d-arabino-heptulosonate 7 phosphate synthase 2 (DHS2, PIF1), which participates in the biosynthesis of aromatic amino acids; clone 56 encoding the small subunit of ribulose bisphosphate carboxylase, which is responsible for carbon fixation and clones 48 and 57 coding for a nicotinate phosphoribosyl transferase and a disulfide oxidoreductase-monooxygenase respectively. Inhibition of these activities will lead to shifts in the metabolic patterns of the cell. This would not be unprecedented as Agrobacterium naturally introduces genes for opine biosynthesis in plant cells that takes away C3 and C4 keto acids and particular amino acids, especially arginine from the metabolic pool. A reduced biosynthesis of aromatic amino acids may diminish the plant defense, as these amino acids are precursors of antimicrobial compounds. Other interactors may also represent proteins involved in plant defense responses. The epithiospecifier protein ESP encoded by clones 22, 33 and 54, is a defense protein that regulates the degradation of glucosinolates by myrosinase upon pathogen attack. This leads to a shift in metabolic products from isothiocianates to epithionitriles (Lambrix et al., 2001). Unfortunately, we could not detect expression in E. coli under the assayed conditions to confirm an interaction of this interesting protein that was identified three times in our screening. The branched chain aminotransferase BCAT4 (clone 44, PIF5) is involved in the synthesis of methionine derived glucosinolates. The unknown proteins encoded by clones 50 and 55 have DCD (development and cell death) and BRE (brain and reproductive organ
expressed) domains, respectively, which are known to be present in proteins involved in the regulation of programmed cell death and in the modulation of stress responses. Also the pirin-like protein encoded by clone 39 (PIF3), may be involved in the regulation of programmed cell death (Orzaez et al., 2001). Finally, interactions were seen with the general translation elongation factor EF2 (clone 53) and with the B3 subunit of the vacular H\(^{+}\) ATPase VHA-B3 (clone 40, PIF4). The latter protein complex is present on intracellular membranes, but also on the plasma membrane and controls the intracellular pH, and thereby a myriad of cellular processes.

Plant cell cultures responded to the presence of Agrobacterium by inducing and down-regulating the expression of a set of genes; among these, several seem to be involved in signal perception, and transduction pathways and defense responses (Ditt et al., 2001; 2006). This indicated that Agrobacterium is recognized as a pathogen by the plant cells, which was also noted by Zipfel et al. (2006) who found that the N-terminal part of the translation elongation factor EF-Tu acted as an elicitor of defense responses in A. thaliana. In fact, recently it was found that downstream defense signaling through the MAP kinase MPK3 leads to the phosphorylation and nuclear targeting of the bZIP transcription factor VIP1, which is suggested to drag along the virulence protein VirE2 as a Trojan horse into the nucleus (Djamei et al., 2007). Agrobacterium thus in fact exploits the induction of the defense response by the plant cells for transformation. It is interesting to note that in the list of genes that are up- and down-regulated by the presence of Agrobacterium (Ditt et al., 2006) the pirin-like gene and the gene for EF2 are present, the products of which are potential targets of the VirF controlled proteolytic system. Research by Veena et al. (2003) revealed that after a primary induction of plant defense/stress related genes, these are turned off and remain down upon transformation with transfer-competent Agrobacterium strains. In the presence of transfer-deficient strains, however, expression of defense related genes is continued. This suggests that general plant defense responses are suppressed during a successful transformation event. This may be accomplished by effector proteins, such as VirF, which are translocated into the plant cells by the bacterium. In our search for host proteins that interact with VirF several proteins associated with plant defense were identified. It remains to be determined whether these proteins are indeed targeted for proteolytic degradation by VirF and how this promotes tumorigenesis.

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