Interaction of the virulence protein VirF of *Agrobacterium tumefaciens* with plant homologs of the yeast Skp1 protein

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The infection of plants by *Agrobacterium tumefaciens* leads to the formation of crown gall tumors due to the transfer of a nucleoprotein complex into plant cells that is mediated by the virulence (*vir*) region–encoded transport system (reviewed in [1–5]). In addition, *A. tumefaciens* secretes the Vir proteins, VirE2 and VirF, directly into plant cells via the same VirB/VirD4 transport system [6], and both assist there in the transformation of normal cells into tumor cells. The function of the 22 kDa VirF protein is not clear. Deletion of the *virF* gene in *A. tumefaciens* leads to diminished virulence [7, 8] and can be complemented by the expression of the *virF* gene in the host plant. This finding indicates that VirF functions within the plant cell [8]. Here, we report that the VirF protein is the first prokaryotic protein with an F box by which it can interact with plant homologs of the yeast Skp1 protein. The presence of the F box turned out to be essential for the biological function of VirF. F box proteins and Skp1p are both subunits of a class of E3 ubiquitin ligases referred to as SCF complexes. Thus, VirF may be involved in the targeted proteolysis of specific host proteins in early stages of the transformation process.

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Results and discussion

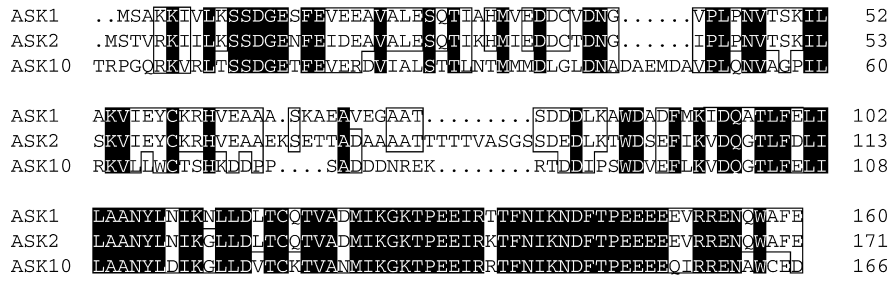
To learn more about the in planta function of VirF, we used the yeast two-hybrid system to search for plant interaction partners. An *Arabidopsis thaliana* cDNA library, constructed from above-ground parts of 2-week-old seedlings and mature flowering plants of the *Arabidopsis thaliana* ecotype Columbia [9], was made in the two-hybrid vector pBI771 [10] and was screened with the *virF* gene cloned in the bait vector pBI770 [10] (pBI770-VirF). *Saccharomyces cerevisiae* strain YPB2 [11], containing the reporter genes *His3* and *lacZ*, was used as a host for the two-hybrid screen. No activity of the *His3* and *lacZ* genes was detected after the transformation of pBI770-VirF alone. It was also not detected in combination with the empty pBI771 prey vector or with pBI771-VirF (data not shown). Thus, VirF does not by itself activate the marker genes and does not interact with itself in the two-hybrid assay. Western blot analysis with VirF polyclonal antibodies confirmed the expression of the Gal4BD-VirF and Gal4AD-VirF fusion proteins in the YPB2 yeast strain (data not shown). Based on these results, we concluded that the pBI770-VirF plasmid would be suitable for screening a cDNA library. Such screens resulted in 16 transformants expressing both reporter genes. From each transformant the cDNA plasmid was rescued and further characterized. Sequence analysis revealed that 14 of the 16 contained the same gene. One of these and the remaining two clones that had unique genes were used again in the two-hybrid assay, which confirmed their specific interaction with VirF (see below).

The 14 cDNA clones, which represented an identical open reading frame (ORF), were found to encode the 18 kDa ASK1 (Arabidopsis Skp1–like) protein. The other two cDNA clones encoded the related 19 kDa ASK2 protein and a new 19 kDa member (ASK10) of the ASK family, which consists of nine previously identified genes. Protein alignment showed that the ASK10 protein was more distant from the other two; it had only 54% and 51% identity with ASK1 and ASK2, respectively (Figure 1). To confirm that the interactions detected by the yeast two-hybrid system were genuine and direct, we mixed purified VirF protein with a glutathione Sepharose 4B matrix bound by either glutathione S-transferase (GST) or by GST-ASK1 or GST-ASK2 fusion protein. After thorough washing, the proteins bound to the matrix were recovered. Immunoblotting showed a clear binding of VirF with the matrix associated with either of the GST-ASK fusion proteins. Some residual binding was found for VirF when GST alone bound the matrix (Figure 2). These biochemical experiments thus confirm the data from the two-hybrid experiments and suggest that there is a direct interaction between VirF and the ASK proteins.

Recently, it was found that the yeast Skp1 protein and its animal and plant homologs, such as the *A. thaliana*

Figure 1

Alignment of the deduced amino acid sequence for ASK1, ASK2, and ASK10. The GenBank accession numbers are, respectively, ATU97020, ATU97021, and AF132729. Similar or identical amino acids are placed in a box; gaps in the alignment are indicated by dots.



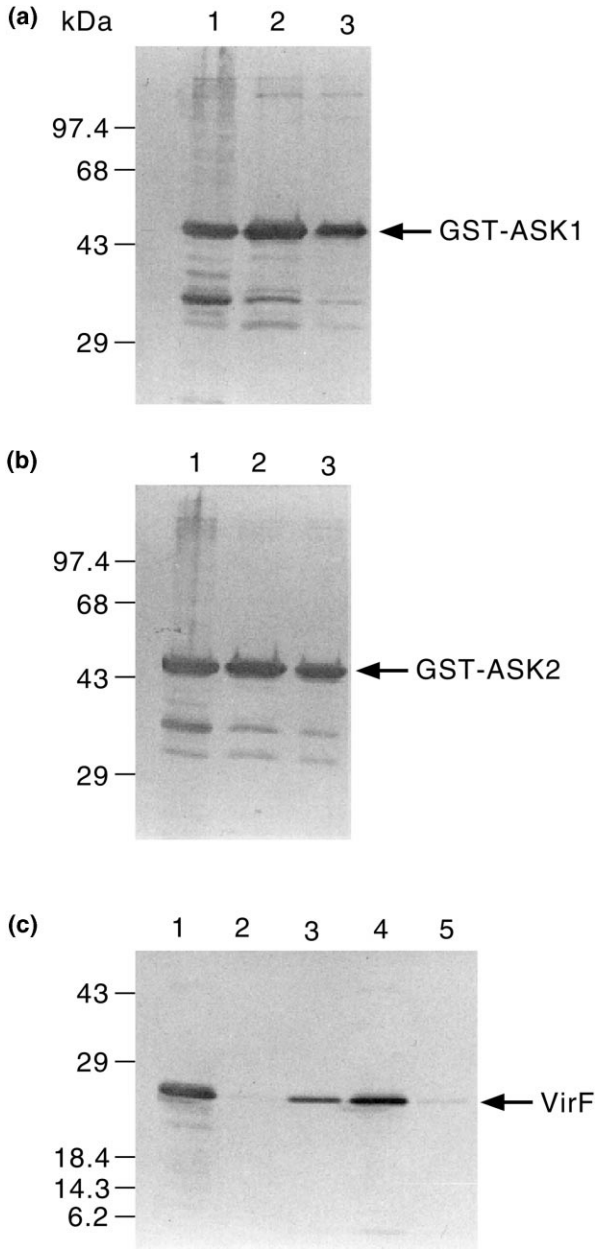
ASK proteins, are subunits of a class of E3 ubiquitin ligases called SCF (Skp1-Cdc53-F box protein) complexes. These complexes target specific proteins for proteolysis and thus play an important regulatory role in processes such as the cell cycle [12–15]. SCF complexes consist of a scaffold protein, Cdc53p or Cullin, to which is bound Cdc34p, which is an ubiquitin-conjugating enzyme; the Rbx1 protein, which assists in the recruitment and activation of Cdc34p; and Skp1p. This latter protein may in turn recruit a variety of so-called F box proteins by binding directly to their F box. The F box proteins act as receptors, which attract specific proteins to the SCF complex for ubiquitination and subsequent proteolysis. The binding of VirF to the ASK proteins suggested that VirF may also contain an F box motif. Comparison of VirF with F box–containing proteins from various species [12] indeed revealed the presence of the conserved leucine and proline residues (amino acids 26, 27, and 38) in VirF. Additionally, the presence of an F box in VirF was confirmed by the ProfileScan program for amino acid positions 20 to 42: KTELLN**LP**DHVLTEVAKR**L**ATNN.

We created an N-terminal deletion, including the deletion of the F box, to confirm that VirF binds ASK1, ASK2, and ASK10 via its F box. For the same reason, we replaced the two most-conserved amino acids of the F box, leucine 26 and proline 27, by alanine. Previous studies have shown that mutation of the conserved leucine and proline residues in the F box protein Cdc4p results in decreased binding of Skp1p [12] and that mutation of the conserved leucine residue in the F box protein SCON2 leads to a loss of function [16]. The YPB2 yeast strain was transformed with either pBI770-VirFΔF-box or pBI770-VirF(LP→AA) alone (data not shown) or in combination with the empty vector pBI771 (Figure 3). As expected, the Gal4BD-VirFΔF-box and Gal4BD-VirF(LP→AA) fusion proteins could not activate transcription of the reporter genes in yeast, as the lack of *lacZ* or *His3* expression indicated. Western blot analysis with VirF polyclonal antibodies showed the expression of the Gal4BD-VirFΔF-box and Gal4BD-VirF(LP→AA) fusion proteins in the YPB2 yeast strain (data not shown). As the control experiments did not show unexpected results, we subsequently used the mutant VirF plasmids in two-hybrid assays with the ASK proteins. In such assays no interaction with ASK1, ASK2, or ASK10 was any longer observed, neither for the VirFΔF-box nor for the VirF(LP→AA) protein, in contrast with the results for the full-length VirF protein (Figure 3a,b). These results indicate that the binding to ASK1, ASK2, and ASK10 is mediated by the F box present in VirF.

To find out whether the presence of an F box is essential for the functioning of VirF in tumorigenesis, we introduced the VirF(LP→AA) mutant allele into an *Agrobacterium virF* deletion mutant. Virulence assays showed that the *Agrobacterium* strains producing the mutant form of VirF instead of wild-type VirF were attenuated in virulence on *Nicotiana glauca* plants, as were *virF* deletion mutants. Figure 3c shows an example of a stem infection. Thus, the presence of a functional F box seems essential for the biological function of VirF. The presence of a functional F box in the *A. tumefaciens* VirF protein is surprising. So far F box proteins have only been found in eukaryotic organisms such as yeast, humans, plants, and fungi as well as in human viruses [12–15], but not in prokaryotes. VirF is the first F box protein identified in bacteria. The finding of a gene for an F box protein on the Ti plasmid of *A. tumefaciens* underscores the remarkable gene content of this genetic element, which includes not only typically eukaryotic genes with eukaryotic expression signals but also prokaryotic genes coding for proteins with eukaryotic features such as the nuclear localization sequence (VirD2, VirE2) and the F box (VirF). Although no direct evidence has been obtained so far, it seems likely that some of this genetic material was introduced into this bacterium by horizontal gene transfer from an eukaryotic organism.

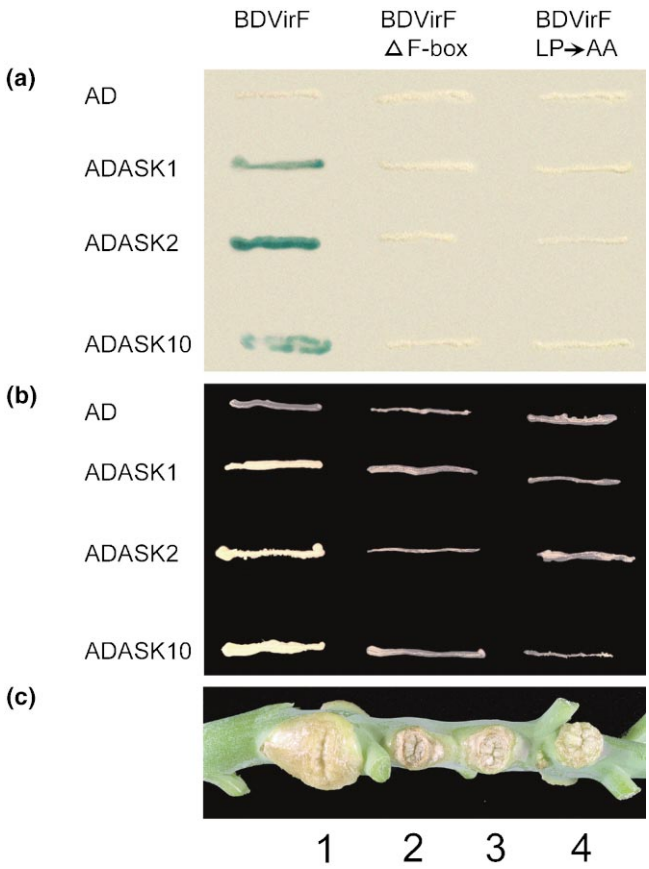
Proteins with similarity to the VirF protein described above are encoded by other types of Ti plasmids as well as by the Ri plasmid of *Agrobacterium rhizogenes* ([17], GenBank accession numbers AF034769 and AP002086). A comparison of the F box regions revealed the presence of the conserved leucine and proline residues in the VirF homologs encoded by the *A. vitis* Ti plasmid and the Ri plasmid. We have shown previously that the VirF homolog

Figure 2



Specific binding of VirF to the ASK proteins. The VirF protein was produced in *E. coli* strain SCS1 and was purified (99.8% pure) by HiTrapQ chromatography, hydroxyapatite chromatography and Superose gel filtration. The *ASK1* and *ASK2* genes from *A. thaliana* were cloned into the GST-fusion vector pGEX-KG (Pharmacia Biotech). The GST-ASK fusion proteins and GST were produced in *E. coli* strain SCS1 and bound to a Glutathione Sepharose 4B matrix. These matrices, loaded with 750 ng GST or GST-ASK, were then incubated with 750 ng VirF. We removed unbound VirF by washing the matrix three times with buffer. We solubilized proteins associated with the matrix in 65 μl total volume by boiling them, and we then separated 15 μl protein sample by SDS-PAGE and identified **(a,b)** ASK or **(c)** VirF after Western blotting. The lanes correspond to the following proteins: (a,b) Lane 1, Lysate of *E. coli* with the GST-ASK protein; Lane 2, GST-ASK protein that was bound to and then recovered from the glutathione matrix; Lane 3, GST-ASK protein that was bound to the matrix, incubated with VirF, and then recovered from the matrix; (c) Lane 1, purified VirF protein; Lane 2, material recovered from the glutathione matrix in the absence of any GST protein; Lane 3, VirF recovered from the matrix associated with GST-ASK1; Lane 4, VirF recovered from the matrix associated with GST-ASK2; Lane 5, material recovered from the matrix associated with GST alone.

Figure 3



The F box of VirF is essential for VirF to bind to the ASK proteins and for its biological function. **(a)** β-galactosidase activity on a filter-lifted yeast streak; **(b)** Growth on medium lacking histidine (+5 mM 3-amino-1′, 2′, 4′-triazole). AD indicates the Gal4 activation domain, BD indicates the Gal4 binding domain. **(c)** Tumorigenicity on *N. glauca* by (1) the control with wild-type *virF*; (2) the *virF* deletion mutant; (3) the *virF* deletion mutant with the *virF* (LP→AA) allele in trans and (4) in cis.

from the Ti plasmid of *A. vitis* can complement *A. tumefaciens virF* deletion mutants for tumor formation on *Nicotiana glauca* [17]. Also, we have observed "extracellular" complementation for tumor formation on *N. glauca* of a *virF* deletion mutant by coinfection with an *Agrobacterium* strain carrying the Ri plasmid of *A. rhizogenes* (our unpublished results). These results can be explained by the presence of a gene encoding a functional VirF homolog on the *A. vitis* Ti plasmid and the Ri plasmid.

The *virF* mutant was initially identified as a mutant with a

mutation in the octopine type Ti plasmid with diminished virulence on tomatoes [18]. Later, *Nicotiana glauca* was found to be the preferred host for studying the effects of *virF* mutations [7]. On other hosts, the role of *virF* in transformation only becomes clear upon the mutation of a second gene, called *virE3* (our unpublished results). The mutation of *virE3* by itself has no or only mild negative effects on the virulence of *Agrobacterium*, but subsequent mutation of *virF* leads to strongly diminished virulence. Apparently, there seems to be some redundancy in the functions mediated by the VirF and VirE3 proteins. The VirE3 protein, like VirE2 and VirF, contains the amino acid motif Arg-Pro-Arg at the C terminus. This motif forms part of the transport signal, which is recognized by the VirB/VirD4 channel, which mediates protein secretion directly from *Agrobacterium* into the plant cell [6]. This suggests that VirE3 is also secreted by *A. tumefaciens* into plant cells via the VirB/VirD4 transport system, as has been shown for VirE2 and VirF [6]. There is no other similarity between the VirF and VirE3 proteins. Also, in contrast to VirF, VirE3 contains putative nuclear localization signal sequences, which suggest a function for VirE3 in the plant cell nucleus. VirE3 may thus compensate for the absence of VirF by an entirely different molecular mechanism of action.

In view of the strong conservation of the Skp1 proteins, we predict that Skp1 proteins of other plant species such as *N. glauca* will interact with VirF similarly to the way in which *A. thaliana* Skp1 homologs do. F box proteins form part of SCF complex ubiquitin ligases and determine which substrate proteins will be targeted for ubiquitination and subsequent proteolysis by the proteasome. Such substrates include promoters and inhibitors of the cell cycle as well as signal transduction components. F box proteins thus play an important regulatory role in the control of cell division and differentiation and, consequently, in the growth and development of eukaryotic organisms. It may be that VirF, as part of an SCF complex, likewise affects the cell cycle. For instance, maintaining cells for an extended period of time in the S-phase may facilitate T-DNA integration. In the plant cell, VirF seems to play a role specifically during the infection by *Agrobacterium* since VirF transgenic plants show no aberrant phenotype (data not shown). The latter may be due to the inherent instability of F box proteins, which are targeted for destruction by ubiquitination during their transient presence in the SCF complex [19]. In recent transport experiments, the delivery of a NLS::Cre::VirF fusion protein into plant cells seemed to occur much more efficiently after deletion of the F box [6]. This may be due not so much to enhanced transport as to enhanced stability after deletion of the F box. A full understanding of the function of VirF will not be possible until the the participation of VirF in an SCF complex is experimentally confirmed and the substrate targeted for proteolysis by the complex has been identified. This substrate must be another direct interaction partner of VirF. However, this has so far not been identified in two-hybrid screens. The reason may be that the substrate needs to be modified in order for the interaction to take place. It is known that many targets must first be phosphorylated and can subsequently interact with the SCF complex [12–15, 20].

The precise mechanism whereby VirF affects tumorigenesis awaits further study, including the identification of the proteins, which are targeted by VirF for proteolysis. But the results obtained so far once more illustrate the sophistication of plant tumor induction by the bacterium *A. tumefaciens*.

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References

1. Kado CI: **Molecular mechanisms of crown gall tumorigenesis.** *Critical Reviews in Plant Sciences* 1991, **10:**1-32.
2. Hooykaas PJJ, Beijersbergen AGM: **The virulence system of *Agrobacterium tumefaciens*.** *Annu Rev Phytopathol* 1994, **32:**157-179.
3. Zupan J, Zambryski P: **The *Agrobacterium tumefaciens* DNA transfer complex.** *Critical Reviews in Plant Sciences* 1997, **16:**279-295.
4. Winans SC: **Two-way chemical signaling in *Agrobacterium*-plant interactions.** *Microbiol Rev* 1992, **56:**12-31.
5. Christie PJ: ***Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria.** *J Bacteriol* 1997, **179:**3085-3094.
6. Vergunst AC, Schrammeijer B, den Dulk-Ras A, de Vlaam CM, Regensburg-Tuïnk TJ, Hooykaas PJ: **VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells.** *Science* 2000, **290:**979-982.
7. Melchers LS, Maroney MJ, den Dulk-Ras A, Thompson DV, van Vuuren HA, Schilperoort RA, Hooykaas PJ: **Octopine and nopaline strains of *Agrobacterium tumefaciens* differ in virulence; molecular characterization of the *virF* locus.** *Plant Mol Biol* 1990, **14:**249-259.
8. Regensburg-Tuïnk AJ, Hooykaas PJ: **Transgenic *N. glauca* plants expressing bacterial virulence gene *virF* are converted into hosts for nopaline strains of *A. tumefaciens*.** *Nature* 1993, **363:**69-71.
9. Kohalmi SE, Nowak J, Crosby WL: **A practical guide to using the yeast 2-hybrid system**. In *Differentially Expressed Genes in Plants: A Bench Manual*. Edited by Harper G and Hansen E. London: Taylor & Francis; 1997:63-82.
10. Kohalmi SE, Reader LJV, Samach A, Nowak J, Haughn GW, Crosby WL: **Identification and characterization of protein interactions using the yeast 2-hybrid system**. In *Plant Molecular Biology Manual M1*. Edited by Gelvin SB and Schilperoort RA. Dordrecht, The Netherlands: Kluwer Academic Publishers; 1998:1-30.
11. Fields S, Song O: **A novel genetic system to detect protein-protein interactions.** *Nature* 1989, **340:**245-246.
12. Bai C, Sen P, Hofmann K, Ma L, Goebl M, Harper JW, Elledge SJ: **SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box.** *Cell* 1996, **86:**263-274.
13. Skowyra D, Craig KL, Tyers M, Elledge SJ, Harper JW: **F-box proteins are receptors that recruit phosphorylated**

substrates to the SCF ubiquitin-ligase complex. *Cell* 1997, **91:**209-219.
14. Feldman RM, Correll CC, Kaplan KB, Deshaies RJ: **A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p.** *Cell* 1997, **91:**221-230.
15. Koepp DM, Harper JW, Elledge SJ: **How the cyclin became a cyclin: regulated proteolysis in the cell cycle.** *Cell* 1999, **97:**431-434.
16. Kumar A, Paietta JV: **An additional role for the F-box motif: gene regulation within the *Neurospora crassa* sulfur control network.** *Proc Natl Acad Sci USA* 1998, **95:**2417-2422.
17. Schrammeijer B, Hemelaar J, Hooykaas PJ: **The presence and characterization of a *virF* gene on *Agrobacterium vitis* Ti plasmids.** *Mol Plant Microbe Interact* 1998, **11:**429-433.
18. Hooykaas PJJ, Hofker M, den Dulk-Ras H, Schilperoort RA: **A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid, and an Ri plasmid by complementation analysis of *Agrobacterium tumefaciens* mutants.** *Plasmid* 1984, **11:**195-205.
19. Galan JM, Peter M: **Ubiquitin-dependent degradation of multiple F-box proteins by an autocatalytic mechanism.** *Proc Natl Acad Sci USA* 1999, **96:**9124-9129.
20. Patton EE, Willems AR, Tyers M: **Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis.** *Trends Genet* 1998, **14:**236-243.