Functional analysis of agrobacterium virulence genes

Niu, X.

Chapter 3

Study on targeted protein degradation under control of the *Agrobacterium* virulence protein VirF in the yeast *Saccharomyces cerevisiae*

Xiaolei Niu, Philippe A Sakalis, Niels van Tol, G Paul H van Heusden and Paul JJ Hooykaas

Department of Molecular and Developmental Genetics, Institute of Biology, Leiden University, Sylviusweg 72, 2333BE Leiden, the Netherlands

Abstract

Agrobacterium tumefaciens is a gram-negative soil bacterium that induces plant tumors by transferring a segment of DNA, called T-DNA, into plant cells. Under laboratory conditions, *Agrobacterium* can also transform many different non-plant organisms such as the yeast *Saccharomyces cerevisiae*. During this process, a number of virulence proteins, including VirF, are translocated into the host cell. VirF contains a putative F-box domain and, according to current theory, in plants and in yeast may induce degradation of the virulence protein VirE2 and the transcription factor VIP1, required for the integration of the T-DNA into hosts chromosomal DNA. In this study, we expressed the *Agrobacterium* virulence proteins VirF and VirE2 and the plant VIP1 protein in *S. cerevisiae* and studied interactions between these proteins and the effect of VirF on the levels of VirE2 and VIP1. To address a possible role of VirF in degradation of host transcription factors or their regulators, we analyzed the effect of *virF* expression on the genome-wide transcription in *S. cerevisiae* using DNA microarrays.

Introduction

Agrobacterium tumefaciens is a gram-negative soil bacterium that can induce plant tumors by transferring a piece of DNA, called T-DNA, into plant cells. Along with the T-DNA a number of virulence proteins, including VirE2, VirF and VirE3, is transferred into the host cell (Vergunst *et al*, 2000). In the plant cytoplasm the T-DNA is coated by VirE2 proteins thus protecting the T-DNA against host nucleases; the VirE2 proteins are bound in turn by the plant VIP1 protein (Anand *et al*, 2007). When VIP1 is phosphorylated it becomes competent to enter into the nucleus. When VIP1 phosphorylation occurs on VIP1 bound to the T-complex, it is thought to help direct the entire T-complex into the nucleus (Djamei *et al*, 2007). The virulence protein VirF contains a putative F box motif by which it can interact with plant orthologs of the yeast Skp1 protein. In this way, VirF may form a Skp-Cullin-F-box protein (SCF-) complex, involved in targeted protein degradation (Schrammeijer *et al*, 2001). It has been reported that the VIP1-VirE2 complex is recognized by VirF resulting in proteasomal degradation of VIP1 and VirE2 in yeast. This protein degradation may be required for uncoating of the T-DNA, enabling integration of the T-DNA into the host chromosomes (Tzfira *et al*, 2004; Lacroix *et al*, 2008). VirF itself may also be subject of proteasomal degradation, which is inhibited by the VirD5 virulence protein (Magori & Citovsky, 2011). However, many details of the VirF-induced protein degradation are still unclear.

Under laboratory conditions, *Agrobacterium* can also transform many different

non-plant organisms such as the yeast *Saccharomyces cerevisiae* (Bundock *et al*, 1995; Piers *et al*, 1996; Soltani *et al*, 2008). The yeast *S. cerevisiae* is an excellent model organism to study fundamental aspects of the *Agrobacterium*-mediated transformation process (Soltani *et al*, 2008; van Attikum *et al*, 2001; van Attikum & Hooykaas, 2003). Therefore, in this study we used *S. cerevisiae* for more detailed analysis of the role of the *Agrobacterium* virulence protein VirF in degradation of VirE2 and VIP1. To this end, we expressed tagged and non-tagged versions of VirF, VirE2 and VIP1 in *S. cerevisiae* and studied the localization, the interactions and the expression levels of these proteins.

The VIP1 protein is a transcription factor. No orthologs of this transcription factor are found in *S. cerevisae*. However, in yeast other transcription factors, i.e. transcription factors having little sequence homology to the VIP1 protein, may have a similar function. These putative transcription factors may be degraded in a VirF-dependent way. Degradation of a transcription factor will most likely result in a reduced transcription of the genes of which expression is regulated by this transcription factor. To investigate whether in *S. cerevisiae* VirF can induce degradation of transcription factors, we expressed VirF in *S. cerevisiae* and analyzed the genome-wide transcription by using DNA microarrays.

Materials and methods

Strains and culture media

The yeast strains used in this study are listed in Table 1. Yeast was cultured in YPD or in MY medium, when required supplemented with uracil, methionine, leucine or histidine (Zonneveld, 1986).

Table 1. Yeast strains used in this study

Yeast Strain	Genotype	Source / Reference
CEN.PK111-32D	*MATa leu2-112*	P. Kötter, Göttingen, Germany.
CEN.PK2-1C	*MATa ura3-52 leu2-112 his3-delta1 trp1-289*	P. Kötter, Göttingen, Germany.
CEN.PK2-1D	*MATalfa ura3-52 leu2-112 his3-delta1 trp1-289*	P. Kötter, Göttingen, Germany.
CEN.PK113-3B	*MATalfa ura3-52 his3-delta1*	P. Kötter, Göttingen, Germany.

426-304YFP-v*irE2* (GG3399)	*MATa ura3-52 leu2-112 his3-delta1 trp1-289::* pRS304[P_{MET17}-YFP-VirE2-T_{CYC1}] *(TRP1)*	This study
435-305CFP-*VIP1* (GG3400)	*MATalfa ura3-52 trp1-289 his3-delta1 leu2-112::* pRS305[P_{MET17}-CFP-VIP1- T_{CYC1}](*LEU2*)	This study
426-304YFP-*virE2* / 435-305CFP-*VIP1* (GG3401)	*MATa/ MATalfa ura3-52/ ura3-52 trp1-289::* pRS304[P_{MET17}-YFP-VirE2-T_{CYC1}] *(TRP1)/ trp1-289 his3-delta1/ his3-delta1 leu2-112::* pRS305[P_{MET17}-CFP-VIP1- T_{CYC1}](*LEU2*) / *leu2-112*	This study
426-305HA-VirE2 (GG3402)	*MATa ura3-52 leu2-112 his3-delta1 trp1-289::* pRS305[P_{MET17}-HA-VirE2- T_{CYC1}] *(LEU2)*	This study
435-305Myc-VIP1 (GG3403)	*MATalfa ura3-52 trp1-289 his3-delta1 leu2-112::* pRS305[P_{MET17}-Myc-VIP1- T_{CYC1}](*LEU2*)	This study
426-305HA-VirE2 / 435-305Myc-VIP1 (GG3404)	*MATa/ MATalfa ura3-52/ ura3-52 trp1-289/ trp1-289 his3-delta1/ his3-delta1 leu2-112::* pRS305[P_{MET17}-HA-VirE2- T_{CYC1}] *(LEU2)/ leu2-112::* pRS305[P_{MET17}-Myc-VIP1- T_{CYC1}](*LEU2*) / *leu2-112*	This study
426-305HA-VirF (GG3405)	*MATa ura3-52 trp1-289 his3-delta1 leu2-112::* pRS305[P_{MET17}-HA-VirF-T_{CYC1}] *(LEU2)*	This study
426-305Myc-VirF (GG3406)	*MATa ura3-52 trp1-289 his3-delta1 leu2-112::* pRS305[P_{MET17}-Myc-VirF-T_{CYC1}] *(LEU2)*	This study
440-305VirF (GG3275)	*MATa leu2*:: pRS305[P_{MET17}-*virF*-T_{CYC1}] (*LEU2*)	This study
440-305 (GG3277)	*MATa leu2*:: pRS305[P_{MET17}-T_{CYC1}] (*LEU2*)	This study

426-305virFΔGFP (GG3407)	*MATa ura3-52 trp1-289 his3-delta1 leu2-112::* pRS305[P_{MET17}-*virF*-T_{CYC1}] (*LEU2*)	This study
426-305ΔGFP (GG3408)	*MATa ura3-52 trp1-289 his3-delta1 leu2-112::* pRS305[P_{MET17}-T_{CYC1}](*LEU2*)	This study

All yeast transformations were carried out using the lithium acetate method (Gietz *et al*, 1995). Yeast strains carrying plasmids were obtained by transformation of the parent strains with the appropriate plasmids followed by selection for uracil and/or histidine prototrophy. 426-304YFP-virE2 was obtained by transformation of CEN.PK2-1C with pRS304[P_{MET17}-YFP-VirE2-T_{CYC1}], followed by selection for tryptophan prototrophy. In this strain YFP-*virE2* under control of the *MET17* promoter and the *CYC1* terminator, was integrated into the yeast chromosomal *TRP1* locus. 435-305CFP-VIP1 was obtained by transformation of CEN.PK2-1D with pRS305[P_{MET17}-CFP-VIP1-T_{CYC1}], followed by selection for leucine prototrophy. The diploid yeast strain 426-304YFP-*virE2* / 435-305CFP-*VIP1* was constructed by mating 426-304YFP-*virE2* with 435-305CFP-*VIP1*, followed by selection for tryptophan and leucine prototrophy. 426-305HA-virE2 was obtained by transformation of CEN.PK2-1C with pRS305[P_{MET17}-HA-VirE2-T_{CYC1}], followed by selection for leucine prototrophy. In this strain 3×HA-*virE2* under control of the *MET17* promoter and the *CYC1* terminator, was integrated into the yeast chromosomal *LEU2* locus. 435-305Myc-VIP1 was obtained by transformation of CEN.PK2-1D with pRS305[P_{MET17}-Myc-VIP1-T_{CYC1}], followed by selection for leucine prototrophy. The diploid yeast strain 426-305HA-*virE2* / 435-305Myc-*VIP1* was constructed by mating 426-305HA-*virE2* with 435-305Myc-*VIP1*, followed by selection of mating cells using a Singer Dissection Microscope MSM. 440-305VirF (GG3275) and 440-305 (GG3277) were obtained by transformation of CEN. PK111-32D with pRS305[P_{MET17}-*virF*-T_{CYC1}], or pRS305[P_{MET17}-T_{CYC1}], respectively, after selection for leucine prototrophy. 426-305HA-VirF and 426-305Myc-VirF were obtained by transformation of CEN.PK2-1C with pRS305[P_{MET17}-HA-VirF-T_{CYC1}] and pRS305[P_{MET17}-Myc-VirF-T_{CYC1}], respectively, followed by selection for leucine prototrophy. 426-virFΔGFP was obtained by transformation of CEN.PK2-1C with pRS305[P_{MET17}-VirF-T_{CYC1}], followed by selection for leucine prototrophy. In this strain VirF under control of the *MET17* promoter and the *CYC1* terminator, was integrated into the yeast chromosomal *LEU2* locus. 426-ΔGFP was obtained by transformation of CEN.PK2-1C with pRS305[PMET17-control-TCYC1], followed by selection for leucine prototrophy. Correct integration was always verified by PCR and/or southern blot analysis.

Plasmid constructions

All plasmids used in this study are listed in Table 2. The *Agrobacterium virF,*

virF(LP-AA) and *virE2* and the *Arabidopsis thaliana* VirE2-interacting protein 1 (*VIP1*) and *ASK1* coding sequences were amplified by PCR on plasmids pSDM3002, pSDM3542, pSDM3163, pSDM3268 and pSDM2061, respectively, using *Phusion*™ *High-Fidelity* DNA Polymerase. For *virF* and *virF(LP-AA)* the oligonucleotides VirF-Fw and VirF-Rev were used, for *virE2* the oligonucleotides VirE2-Fw — VirE2-Rev1 and VirE2-Fw — VirE2-Rev2, for *VIP1* the oligonucleotides Vip1-Fw — Vip1-Rev and for *ASK1* the oligonucleotides ASK1-Fw and ASK1-Rev (Table 3). The *S. cerevisiae SKP1* and *CDC53* coding sequences were PCR amplified on genomic DNA isolated from yeast strain CEN.PK113-3B, using oligonucleotide combinations Skp1-Fw1 — Skp1-Rev1 or Skp1-Fw2 — Skp1-Rev2 or Skp1-Fw2 —Skp1-Rev3 and Cdc53-Fw — Cdc53-Rev, respectively (Table 3). PCR fragments were inserted into the PCR Blunt II TOPO vector (Invitrogen) or CloneJET™ PCR Cloning vector (Fermentas) as recommended by the manufacturer, yielding pTOPO[virF], pJET1.2[virF(LP-AA)], pJET1.2[virE2-A], pJET1.2[virE2-B], pTOPO[VIP1], pJET1.2[ASK1], pJET1.2[SKP1-A] , p—JET1.2[Skp1-B] pJET1.2[Skp1-C]and pTOPO[CDC53].

Table 2. Plasmids

Plasmid	Properties	Source / Reference
pSDM3002	pUC19 with a 2.0 kb *Sac*I fragment with *virF*	(García-Rodríguez, Fernando *et al*, 2006)
pSDM3542	pUC19 with a *Bam*HI-*Xho*I fragment with HA-tagged *virF* (LP-AA)	E. Jurado-Jácome, Ph.D. thesis
pSDM3163	pBluescript with a fragment with the virE operon	D. Schneider, unpublished
pSDM3268	pBBR6 withVIP1 fused to the VirF transport signal	C. Michielse, unpublished
pSDM2061	pGEM-T Easy containing ASK1 amplified from a Arabidopsis cDNA library	P. Bundock unpublished
pTOPO[virF]	PCR Blunt II TOPO containing virF flanked by *Bam*HI and *Sal*I restriction sites.	This study
pJET1.2[virF(LP-AA)]	CloneJET™ PCR Cloning vector with *virF*(LP-AA) flanked by *Bam*HI and *Sal*I restriction sites.	This study
pJET1.2[virE2-A]	CloneJET™ PCR Cloning vector with *virE2* (A) flanked by *Spe*I and *Xma*I restriction sites.	This study
pJET1.2[virE2-B]	CloneJET™ PCR Cloning vector with *virE2* (B) flanked by *Spe*I and *Xma*I restriction sites.	This study
pTOPO[VIP1]	PCR Blunt II TOPO containing Arabidopsis VIP1 flanked by *Bam*HI and *Sal*I restriction sites.	This study

pJET1.2[ASK1]	CloneJET™ PCR Cloning vector with *ASK1* flanked by *Bam*HI and *Eco*RI restriction sites.	This study
pJET1.2[SKP1-A]	CloneJET™ PCR Cloning vector with *SKP1* flanked by *Bam*HI and *Sal*I restriction sites.	This study
pJET1.2[Skp1-B]	CloneJET™ PCR Cloning vector with *SKP1* flanked by *Spe*I and *Xho*I restriction sites.	This study
pJET1.2[Skp1-C]	CloneJET™ PCR Cloning vector with *SKP1* flanked by *Spe*I and *XmaI* restriction sites.	This study
pTOPO[CDC53]	PCR Blunt II TOPO containing *CDC53* flanked by *Spe*I and *Xma*I restriction sites.	This study
pTOPO[virF]-2	PCR Blunt II TOPO containing virF flanked by *Spe*I and *Sal*I restriction sites.	This study
pTOPO[virF]-3	PCR Blunt II TOPO containing virF flanked by *Spe*I and *Xma*I restriction sites.	This study
pJET1.2[virF(LP-AA)]-2	CloneJET™ PCR Cloning vector with *virF*(LP-AA) flanked by *SpeI* and *Sal*I restriction sites.	This study
pJET1.2[virF(LP-AA)]-3	CloneJET™ PCR Cloning vector with *virF*(LP-AA) flanked by *SpeI* and *XmaI* restriction sites.	This study
pUG34	Centromeric plasmid to make N-terminal GFP fusions under control of the *MET17* (alias *MET25*) promoter and *CYC1* terminator. *HIS3* marker.	U. Güldener and J.H. Hegemann
pUG34[virF] (pSDM3357)	Centromeric plasmid with GFP-*virF* under control of the *MET17* promoter and *CYC1* terminator. *HIS3* marker.	This study
pUG34[SKP1] (pSDM3359)	Centromeric plasmid with GFP-SKP1 under control of *MET17* promoter and *CYC1*. *HIS3* marker.	This study
pUG34[VIP1] (pSDM3361)	Centromeric plasmid with GFP-VIP1 under control of *MET17* promoter and *CYC1*. *HIS3* marker.	This study
pUG34CFP[VirE2]	Centromeric plasmid with CFP-VirE2 under control of *MET17* promoter and *CYC1* terminator. *HIS3* marker.	P.A. Sakalis
pUG34-HA (pSDM3362)	Centromeric plasmid to make N-terminal 3×HA fusions under control of the *MET17* promoter and *CYC1* terminator. *HIS3* marker.	This study
pUG34-HA[VirF] (pSDM3363)	Centromeric plasmid with 3×HA-VirF under control of *MET17* promoter and *CYC1*. *HIS3* marker.	This study
pUG34-HA[virE2] (pSDM3365)	Centromeric plasmid with 3×HA-virE2 under control of *MET17* promoter and *CYC1* terminator. *HIS3* marker.	This study

pUG34-HA[ASK1] (pSDM3367)	Centromeric plasmid with 3×HA-Ask1 under control of *MET17* promoter and *CYC1*. *HIS3* marker.	This study
pUG34ΔGFP (pSDM3369)	Centromeric plasmid with the *MET17* promoter and *CYC1* terminator. *HIS3* marker. Made by removal of GFP from pUG34.	This study
pUG34ΔGFP[virF] (pSDM3370)	Centromeric plasmid with untagged VirF under control of the *MET17* promoter and *CYC1* terminator. *HIS3* marker.	This study
pUG34ΔGFP[ASK1] (pSDM3371)	Centromeric plasmid with ASK1 under control of the *MET17* promoter and *CYC1* terminator. *HIS3* marker.	This study
pUG36CFP	Centromeric plasmid to make N-terminal CFP fusions under control of the *MET17* promoter and *CYC1* terminator. *URA3* marker.	(van Hemert *et al*, 2003)
pUG36YFP	Centromeric plasmid to make N-terminal YFP fusions under control of the *MET17* promoter and *CYC1* terminator. *URA3* marker.	M. Miedema and G.P.H. van Heusden, unpublished results
pUG36YFP[virE2]	Centromeric plasmid with YFP-VirE2 under control of MET17 promoter and *CYC1* terminator. *URA3* marker.	P.A. Sakalis
pUG36-Myc (pSDM3372)	Centromeric plasmid to make N-terminal 3×Myc fusions under control of the *MET17* promoter and *CYC1* terminator. *URA3* marker.	This study
pUG36-Myc[VirF] (pSDM3373)	Centromeric plasmid with 3×Myc-VirF under control of *MET17* promoter and *CYC1* terminator. *URA3* marker.	This study
pUG36-Myc[VIP1] (pSDM3374)	Centromeric plasmid with 3×Myc-VIP1 under control of *MET17* promoter and *CYC1* terminator. *URA3* marker.	This study
pUG36CFP[VIP1] (pSDM3377)	Centromeric plasmid with CFP-VIP1 under control of *MET17* promoter and *CYC1*. *URA3* marker.	This study
pUG36[VIP1] (pSDM3378)	Centromeric plasmid with GFP-VIP1 under control of *MET17* promoter and *CYC1*. *URA3* marker.	This study
pRS304	Yeast integration plasmid. *TRP1* selection marker.	(Gietz & Sugino, 1988)
pRS305	Yeast integration plasmid. *LEU2* selection marker.	(Gietz & Sugino, 1988)
pRS304[P_{MET17}-YFP-VirE2- T_{CYC1}] (pSDM3379)	pRS304 containing YFP-*virE2* under control of *MET17* promoter and *CYC1* terminator. *TRP1* marker, integration plasmid.	This study

pRS305[P_{MET17}-HA-VirF-T_{CYC1}] (pSDM3382)	pRS305 containing 3×HA -*virF* under control of *MET17* promoter and *CYC1* terminator. *LEU2* marker, integration plasmid.	This study
pRS305[P_{MET17}-Myc-VirF-T_{CYC1}] (pSDM3383)	pRS305 containing 3×Myc -*virF* under control of *MET17* promoter and *CYC1* terminator. *LEU2* marker, integration plasmid.	This study
pRS305[P_{MET17}-T_{CYC1}] (pSDM3386)	pRS305 containing *MET17* promoter and *CYC1* terminator. *LEU2* marker, integration plasmid.	This study
pRS305[P_{MET17}-VirF-T_{CYC1}] (pSDM3387)	pRS305 containing *virF* under control of *MET17* promoter and *CYC1* terminator. *LEU2* marker, integration plasmid.	This study
pRS305[P_{MET17}-HA-VirE2- T_{CYC1}] (pSDM3389)	pRS305 containing 3×HA-VirE2 under control of *MET17* promoter and *CYC1* terminator. *LEU2* marker, integration plasmid.	This study
pRS305[P_{MET17}-Myc-VIP1- T_{CYC1}] (pSDM3390)	pRS305 containing 3×Myc-VIP1 under control of *MET17* promoter and *CYC1* terminator. *LEU2* marker, integration plasmid.	This study
pRS305[P_{MET17}-CFP-VIP1- T_{CYC1}] (pSDM3391)	pRS305 containing CFP-*VIP1* under control of *MET17* promoter and *CYC1* terminator. *LEU2* marker, integration plasmid.	This study
pQE30	Plasmid allowing expression of 6×His-tagged proteins under control of the T5 bacteriophage promoter in *E. coli*.	Qiagen
pQE30[virF] (pSDM3436)	Plasmid with 6×His-*virF* under control of the T5 bacteriophage promoter, for expression in *E. coli*.	This study
pQE30[SKP1] (pSDM3438)	Plasmid with 6×His-*SKP1* under control of the T5 bacteriophage promoter, for expression in *E. coli*.	This study
pQE30[ASK1] (pSDM3439)	Plasmid with 6×His-*ASK1* under control of the T5 bacteriophage promoter, for expression in *E. coli*.	This study
pGEX-KG	Plasmid allowing expression N-terminal glutathione S-transferase (GST) fusion protein in *E.coli* under control of the *tac* promoter.	(Guan & Dixon, 1991)
pGEX-KG[virF] (pSDM3441)	Plasmid allowing expression of GST-VirF under control of the *tac* promoter.	This study
pGEX-KG [virF(LP-AA)] (pSDM3442)	Plasmid allowing expression of GST-VirF(LP-AA) under control of the *tac* promoter.	This study
pUG34VN	Centromeric plasmid to make N-terminal fusions with the N-terminal Venus fragment under control of the *MET17* promoter and *CYC1* terminator. *HIS3* marker.	P.A. Sakalis, unpublished

pUG34VC	Centromeric plasmid to make N-terminal fusions with the C-terminal Venus fragment under control of the *MET17* promoter and *CYC1* terminator. *HIS3* marker.	P.A. Sakalis unpublished
pUG35VN	Centromeric plasmid to make C-terminal fusions with the N-terminal Venus fragment under control of the *MET17* promoter and *CYC1* terminator. *URA3* marker.	P.A. Sakalis unpublished
pUG35VC	Centromeric plasmid to make C-terminal fusions with the N-terminal Venus fragment under control of the *MET17* promoter and *CYC1* terminator. *URA3* marker.	P.A. Sakalis unpublished
pUG34VC[virF] (pSDM3450)	Centromeric plasmid with *VC-virF* under control of the *MET17* promoter and *CYC1* terminator. *HIS3* marker.	This study
pUG34VC[SKP1] (pSDM3458)	Centromeric plasmid with *VC-SKP1* under control of the *MET17* promoter and *CYC1* terminator. *HIS3* marker.	This study
pUG34VC[VIP1]	Centromeric plasmid with *VC-VIP1* under control of the *MET17* promoter and *CYC1* terminator. *HIS3* marker.	P.A. Sakalis
pUG35VN[virF] (pSDM3451)	Centromeric plasmid with *virF-VN* under control of the *MET17* promoter and *CYC1* terminator. *URA3* marker.	This study
pUG35VN[virF(LP-AA] (pSDM3455)	Centromeric plasmid with *virF-VN* under control of the *MET17* promoter and *CYC1* terminator. *URA3* marker.	This study
pUG35VN[SKP1] (pSDM3459)	Centromeric plasmid with *SKP1-VN* under control of the *MET17* promoter and *CYC1* terminator. *URA3* marker.	This study
pUG35VN[virE2]	Centromeric plasmid with *virE2-VN* under control of the *MET17* promoter and *CYC1* terminator. *URA3* marker.	P.A. Sakalis
pUG35VN[CDC53] (pSDM3463)	Centromeric plasmid with *CDC53-VN* under control of the *MET17* promoter and *CYC1* terminator. *URA3* marker.	This study
pMVHis	Plasmid allowing expression of 6×His-tagged proteins under the control of galactose inducible *GAL1* promoter for expression in *S. cerevisiae*.	(van Hemert *et al*, 2003)
pMVHis[SKP1] (pSDM3475)	Plasmid with 6×His-*SKP1* under the control of the galactose inducible promoter, for expression in *S. cerevisiae*.	This study

pMVHis[ASK1] (pSDM3476)	Plasmid with 6×His-*ASK1* under the control of the galactose inducible promoter, for expression in *S. cerevisiae*.	This study
pJS95	2 µ plasmid with ASK1 under control of the PGK1 promoter and terminator. *TRP1* marker.	(Schouten *et al*, 2000)
pCR2.1-3xMyc-3x HA (pSDM3485)	pCR 2.1 TOPO-TA containing 3×Myc-3×HA sequences flanked by *XbaI* and *BglII* restriction sites synthesized by Eurofins.	This study

Table 3. Oligonucleotides for PCR amplification

Name	**Sequence**	**Restriction site**
VirF-Fw	5'-AA*GGATCC*ATGAGAAATTCGAGTTTGCGTGATG-3'	*BamHI*
VirF-Rev	5'-AAGTCGACTCATAGACCGCGCGTTGATCGAGGT-3'	*SalI*
VirE2-Fw	5' GGACTAGTATGGATCTTTCTGGCAA-3'	*SpeI*
VirE2-Rev1	5' CCCCCGGGTCAAAAGCTGTTGACGC-3'	*XmaI*
VirE2-Rev2	5'CCCCCGGGAAAGCTGTTGACGCTTT-3'	*XmaI*
VIP1-Fw	5'-AAGGATCCATGGAAGGAGGAGGAAGAGGACC-3'	*BamHI*
VIP1-Rev	5'AAGTCGACTCAGCCTCTCTTGGTGAAATCCA-3'	*SalI*
ASK1-Fw	5'AAAGAATTCATGTCTGCGAAGAAGATTGTGT-3'	*BamHI*
ASK1-Rev	5'AAAGGATCCTCATTCAAAAGCCCATTGGTTC-3'	*EcoRI*
Skp1- Fw1	5'AAAGGATCCATGGTGACTTCTAATGTTGT-3'	*BamHI*
Skp1-Rev1	5'AAAGTCGACCTAACGGTCTTCAGCCCATT-3'	*SalI*
VirF-Fw2	5'-AAACTAGTATGAGAAATTCGAGTTTGCGTG-3'	*SpeI*
VirF-Rev2	5'-AACCCGGGTAGACCGCGCGTTGATCGAGGT-3'	*XmaI*
Skp1-Fw2	5'-AAACTAGTATGGTGACTTCTAATGTTGTCC-3'	*SpeI*
Skp1-Rev2	5'-AACTCGAGCTAACGGTCTTCAGCCCATTCAT-3'	*XhoI*
Skp1-Rev3	5'-AACCCGGGACGGTCTTCAGCCCATTCAT-3'	*XmaI*
Cdc53-Fw	5'-AAACTAGTATGTCTGAGACTCTGCCTAGAT-3'	*SpeI*
Cdc53-Rev	5'-AACCCGGGAGCAAGGTAAGCATACGATTCAC-3'	*XmaI*

DNA fragments containing *virF* or *virF(LP-AA)* were obtained by digestion of pTOPO[virF] or pJET1.2[virF(LP-AA)] with *Bam*HI and *Sal*I restriction enzymes and were cloned into pUG34, pQE30 and pGEX-KG, after digestion with the same restriction enzymes, producing pUG34[virF] , pQE30[virF], pGEX-KG[virF] and

pGEX-KG[virF(LP-AA)], respectively. pUG34[virF] expresses GFP-VirF under the control of *MET17* promoter and *CYC1* terminator. pQE30 [virF] expresses VirF as an N-terminal 6 ×histidine fusion protein under control of the T5 bacteriophage promoter in *E. coli.* pGEX-KG[virF] and pGEX-KG[virF(LP-AA)] express VirF and VirF(LP-AA), respectively, as a N-terminal glutathione S-transferase (GST) fusion protein in *E. coli*, under the control of the *tac* promoter.

DNA fragments containing VIP1 were obtained by digestion of pTOPO[VIP1] with *Bam*HI and *Sal*I restriction enzymes and were cloned into pUG34, pUG36CFP and pUG36-Myc, after digestion with the same restriction enzymes, producing pUG34[VIP1], pUG36CFP[VIP1] and pUG36-Myc[VIP1], respectively. DNA fragments containing VIP1 was obtained by digestion pUG34[VIP1] with *BspE1* and *SalI* restriction enzyme and were cloned into pUG36-Myc, digested with the same restriction enzyme, producing pUG36[VIP1]. These plasmids express GFP-VIP1, CFP-VIP1 and 3×Myc-VIP1, respectively, under control of *MET17* promoter and *CYC1* terminator. pUG34[SKP1] and pQE30[SKP1] were obtained by ligation of a DNA fragment with *SKP1* obtained by digestion of pJET1.2[SKP1-A] with *Bam*HI and *Sal*I restriction enzymes, into pUG34 and pQE30, respectively, digested with the same restriction enzymes. Plasmid pQE30 [SKP1] expresses Skp1 as an N-terminal 6 ×histidine fusion protein under control of the T5 bacteriophage promoter in *E. coli*. A DNA fragment containing *ASK1* was obtained by digestion of pJET1.2[ASK1] with *Bam*HI and *Eco*RI restriction enzymes and was cloned into pUG34-HA and pUG34ΔGFP, digested with the same restriction enzyme, producing pUG34-HA[ASK1] and pUG34ΔGFP[ASK1], respectively. Plasmid pQE30[ASK1] was obtained by transfer of a DNA fragment with *ASK1* from pUG34-HA[ASK1] to pQE30 after digestion with *BamHI* and *HindIII*.

pUG34-HA[VirF] and pUG36-Myc[VirF] were obtained by ligation of a DNA fragment with *virF* obtained by digestion of 34GFP[VirF] with *BamHI* and *SalI* restriction enzymes, into pUG34HA and pUG36Myc, respectively, digested with the same restriction enzymes. pUG34-HA[VirF] and pUG36-Myc[VirF] express 3×HA-VirF and 3×Myc-VirF under the control of *MET17* promoter and *CYC1* terminator.

pMVHis[ASK1] was obtained by ligation of a DNA fragment with *ASK1* obtained by digestion of 34HA[ASK1] with *BamHI* and *EcoRI* restriction enzymes, into pMVHis digested with *BglII* and *EcoRI* restriction enzymes. pMVHis[SKP1] was obtained by ligation of a DNA fragment with *SKP1* obtained by digestion of 34GFP[SKP1] with *BamHI* and *SalI* restriction enzymes, into pMVHis digested with *BglII* and *XhoI* restriction enzymes. Plasmid pMVHis[ASK1] and pMVHis[SKP1] express ASK1 or Skp1 as an N-terminal 6 ×histidine fusion protein under control of the galactose inducible *GAL1* promoter in *S. cerevisiae*.

pUG36-Myc was obtained by replacement of a *Xba*I-*Bam*HI fragment containing GFP in pUG36 with a *Xba*I-*BamHI* fragment from plasmid pCR2.1-3×Myc-3× HA, containing the 3×Myc epitope. Plasmid pCR2.1-3×Myc-3×HA, containing the 3×Myc and 3×HA epitope sequences, was synthesized by Eurofins (sequence of tags: *TCTAGA*ATGGGTGAACAAAAGTTGATTTCTGAAGAAGATTTGAACGGTGAACAAAAA GCTAATCTCCGAGGAAGACTTGAACGGTGAACAAAAATTAATCTCAGAAGAAGACTTG AACGGATCA*GGATCC*GAATTC*ACTAGT*ATGATCTTTTACCCATACGATGTTCCTGACTAT GCGGGCTATCCCTATGACGTCCCGGACTATGCAGGATATCCATATGACGTTCCAGATTAC GCTGCTCAGTGC*ACTAGT*AGATCT).

Plasmid pUG34-HA was obtained in a similar way by replacement of an *XbaI-SpeI* fragment containing GFP in pUG34 with a *SpeI* fragment of pCR2.1-3×Myc3×HA, containing the 3×HA epitope. Plasmid pUG36YFP[virE2] was obtained by transfer of a DNA fragment with *virE2* from pJET1.2 [virE2-A] to pUG36YFP after digestion with *Spe*I and *Xma*I. In this plasmid *virE2* is N-terminally fused to YFP and its transcription is controlled by the *MET17* promoter and *CYC1* terminator. pUG34-HA[virE2] was made by replacement of a *XbaI-SpeI* fragment containing CFP in pUG34CFP[VirE2] by a *SpeI* fragment from pCR2.1-3×Myc-3× HA, containing the 3×HA epitope.

To allow integration into the yeast genome, a fragment with YFP-VirE2 including the *MET17* promoter and *CYC1* terminator was released from pUG36YFP[VirE2] by digestion with *Bsp*E1 and *Kpn*I and ligated into pRS304 digested with *Xma*I and *Kpn*I, resulting in pRS304[P_{MET17}-YFP-VirE2-T_{CYC1}]. To allow integration of CFP-VIP1 into the yeast genome, a fragment with CFP-VIP1 including the *MET17* promoter and *CYC1* terminator, was released from pUG36CFP[VIP1] by digestion with *Bsp*E1 and *Eag*I and ligated into pRS305 digested with *Xma*I and *Eag*I, resulting in pRS305[P_{MET17}-CFP-VIP1-T_{CYC1}]. In a similar way, the plasmids pRS305[P_{MET17}-HA-virE2-T_{CYC1}] and pRS305[P_{MET17}-Myc-VIP1-T_{CYC1}] were made by transferring 3×HA-VirE2 and 3×Myc-VIP1 and the *MET17* promoter and *CYC1* terminator from pUG34-HA[virE2] and pUG36-Myc[VIP1], respectively, into pRS305. The plasmids pRS305[P_{MET17}-HA-virF-T_{CYC1}] and pRS305[P_{MET17}-Myc-virF-T_{CYC1}] were made by transferring 3×HA-*virF* and 3×Myc-*virF* and the *MET17* promoter and *CYC1* terminator from pUG34-HA[VirF] and pUG36-Myc[VirF], respectively, into pRS305.

To remove sequences coding for GFP, pUG34[virF] was digested with *Xba*I and religated, resulting in pUG34ΔGFP[virF]. The control plasmid pUG34ΔGFP was made by digestion of pUG34 followed by religation. To allow integration into the yeast genome, the *virF* gene including the *MET17* promoter and *CYC1* terminator was released from pUG34ΔGFP[virF] by digestion with *Bsp*E1 and *Eag*I and ligated into pRS305 digested with *Xma*I and *Eag*I, resulting in pRS305[P_{MET17}-virF-T_{CYC1}] . In a similar way the control plasmid pRS305[P_{MET17}-T_{CYC1}] was made by transfer of

the *MET17* promoter and *CYC1* terminator from pUG34ΔGFP into pRS305.

For the construction of plasmids for BiFC analysis, the *virF* coding sequence was amplified by PCR on plasmid pTOPO[virF]/ pJET1.2[virF(LP-AA)] with oligonucleotides VirF-Fw2 and VirF-Rev for N-terminal fusions and with VirF-Fw2 and VirF-Rev2 for C-terminal fusions using *Phusion*™ *High-Fidelity* DNA Polymerase. The PCR products were gel purified and inserted into the PCR Blunt II TOPO/ pJET1.2 vector yielding pTOPO[virF]-2/ pJET1.2[virF(LP-AA)]-2 and TOPO[virF]-3/ pJET1.2[virF(LP-AA)]-3, respectively. pUG34VC[virF]/ pUG34VC[virF(LP-AA)] encoding VC-VirF/ VC-VirF(LP-AA) were created by inserting a *Spe*I–*Sal*I fragment with *virF* from pTOPO[virF]-2/ pJET1.2[virF(LP-AA)]-2 into pUG34VC digested with *SpeI* and *XhoI*. pUG35VN[virF] / pUG35VN[virF(LP-AA)] encoding VirF-VN /VirF(LP-AA)-VN were created by inserting a *Spe*I–*Xma*I fragment with *virF* from pTOPO[virF]-3/ pJET1.2[virF(LP-AA)]-3 into pUG35VN. pUG34VC[SKP1] encoding VC-Skp1 was created by inserting a *Spe*I–*Xho*I fragment with *SKP1* from pJET1.2[Skp1-B] into pUG34VC. pUG35VN[SKP1] encoding SKP1-VN was created by inserting a *Spe*I–*Xma*I fragment with *SKP1* from pJET[SKP1-C] into pUG35VN. pUG35VN[CDC53] encoding Cdc53-VN was created by inserting a *Spe*I–*Xma*I fragment with *CDC53* from pTOPO[CDC53] into pUG35VN. pUG35VN[virE2] encoding VirE2-VN was created by inserting a *SpeI–XmaI* fragment with *virE2* form pJET1.2[virE2-B] into pUG35VN. pUG34VC[VIP1], encoding VC-VIP1 was created by inserting a *SpeI–XhoI* fragment with *VIP1* form pUG36CFP[VIP1] into pUG34VC.

All PCR fragments were verified by sequencing before using them for plasmid constructions. Correct ligation was checked by restriction analysis and by sequencing the junctions between the ligated plasmids and DNA fragments.

Protein extraction and Western blotting

Yeast strains were grown in MY liquid medium supplemented with the appropriate nutrients. Cells were harvested by centrifugation at 4000 rpm for 5 min and washed once with ice-cold MilliQ water. The pellets were then frozen in liquid nitrogen and stored at -80°C for future use. For Western blotting protein samples were applied onto precast polyacrylamide gels (any kD resolving gel, Bio-Rad) and transferred to PVDF Western blotting membranes (Roche). Blots were probed with the anti-HA-Peroxidase antibody (Roche High Affinity 3F10), the anti-Myc antibody (Myc-Tag 71D10 Rabbit mAb, Cell Signaling) or the anti-6×His antibody (His-probe-HRP, SC-8036 Santa Cruz). Signals were detected using the Western blotting Lightning Plus substrate (Perkin Elmer) and the Geliance 600 Imager.

Co-purification experiments

6×His-ASK1 and 6×His-Skp1 proteins were purified form *E. coli* XL1-blue cells

carrying pQE30[ASK1] and pQE30[SKP1], respectively, using Ni-NTA agarose (Qiagen) and elution with 200 mM Imidazole according to manufacturer's protocol. GST, GST-VirF and GST-VirF (LP-AA) were expressed in *E.coli* BL21(DE3) and immobilized on Glutathione HiCap Matrix (Qiagen) beads, according to the manufacture's protocol and washed 4 times with buffer TN1. GST-, GST-VirF- and GST-VirF(LP-AA)-loaded beads were incubated with purified 6×His-ASK1 and 6×His-Skp1 in phosphate-buffered saline buffer containing 1× protease inhibitor cocktail (Roche) and rotated end to end for 2 hours at 4°C. After four washes with buffer TN1, bound proteins were eluted with buffer TN2 (provided in Glutathione HiCap Matrix Kit). 6×His-ASK1 and 6×His-Skp1 were detected by immunoblotting with anti-His antibody (Santa Cruz). GST, GST-VirF and GST-VirF (LP-AA) were detected by Coomassie blue staining.

To study the interaction between VirF and Skp1 in yeast, strains harbouring plasmid pMVHis-ASK1 or pMVHis-SKP1 were inoculated in MY glucose medium supplemented with tryptophan plus histidine and grown until the OD reached 0.6. Then, yeast cells were centrifuged and washed with MilliQ water to remove residual glucose, and transferred to MY-medium containing 1 % (w/v) galactose instead of glucose for sub-culture for an additional 4 hours to induce the expression of 6×His-ASK1 or 6×His-Skp1. Yeast cells were harvested by centrifugation and homogenized in 50 mM sodium phosphate (pH 8.0)/250 mM NaCl containing protease inhibitors (Roche, complete protease inhibitor cocktail) using the FastPrep apparatus. The homogenates were centrifuged for 10 min at 13,000 rpm at 4°C twice. 6×His-ASK1 or 6×His-Skp1 were purified by addition of 200 ul Ni-NTA agarose (Qiagen) suspension to 1 ml of the extract. After incubation for 2 hrs at 4 °C the raisin was collected by centrifugation, washed 4 times and 6×His-ASK1/6×His-SKP1 proteins were eluted with 100 ul elution buffer(50 mM Na-Phosphate, 250 mM NaCl, 0.01% Tween-20, pH 8.0) containing 300 mM Imidazole. Western blotting was performed to detect whether Myc-VirF or HA-VirF was co-purified with 6×His-ASK1 or 6×His-SKP1 using the anti-Myc (Myc-Tag -71D10- Rabbit mAb, Cell Sigaling), anti-HA (Anti-HA-Peroxidase, High Affinity 3F10, Roche) and anti-His (His-probe H-3: sc-8036, Santa Cruz Biotechnology) antibodies.

Confocal microscopy

Yeast cells were grown in MY medium supplemented with the appropriate nutrients and were analyzed using a Zeiss LSM5 Exciter confocal microscope. For BiFC excitation was at 514 nm and emission was detected at 530-600 nm. Microscopic images were analyzed using ImageJ software (Abràmoff *et al*, 2004).

Flow cytometry

Yeast cells were grown in MY medium supplemented with the appropriate amino acids and diluted 25-fold before flow cytometry. The Guava EasycyteTM system

from Merck MILLIPORE was used and data were analyzed with CytoSoftTM software. A 488 nm laser and a 510-540 nm band pass filter were used to detect fluorescence. For each strain at least twleve independent transformants were analyzed. For each transformant 5000 cells were analyzed.

Microarray analysis

SD medium (200 ml) in 2 liter Erlenmeyer flasks was inoculated with strains GG3275 (VirF), or GG3277 (control) by addition of aliquots from overnight cultures in SD medium till A_{620} of 0.05. Then, the cultures were incubated at 30 °C under constant shaking at 250 rpm until an A_{620} of 0.60 was reached. The cultures were rapidly frozen by pouring them into liquid nitrogen. Pieces of the frozen cultures were thawed on ice and RNA was isolated using an Ambion RiboPure™-Yeast RNA isolation kit. Three of the four RNA samples from each strain were labeled and analyzed using Affymetrix GeneChip Yeast Genome 2.0 Arrays by ServiceXS (Leiden, the Netherlands). Briefly, from each RNA sample 100 ng was used to synthesize Biotin-labeled cRNA with the help of an Affymetrix 3' IVT-express Labeling Kit. The cRNA was fragmented and 3 µg of fragmented cRNA was utilized for the hybridization of the GeneChips using the GeneChip Hybridization, Wash and Stain Kit (Affymetrix). The Affymetrix software program GeneChip Command Console (v3.1) was used to operate the staining, washing and scanning of the GeneChips. Data were analyzed using the Rosetta Resolver (v7.2) and Bioconductor programs (Gentleman *et al*, 2004).

Results

Alignment and localization of VirF protein

Previously, in our group it was discovered that virulence protein VirF contains an putative F-box motif by which it can interact with the plant orthologs of the yeast Skp1 protein and also that the protein can interact with a variety of host proteins (Jurado, 2011; Schrammeijer *et al*, 2001).

As can be seen from the alignment shown in Figure 1, the VirF protein and especially the putative F-box motif are well conserved in the different wild type *Agrobacterium* strains.

To understand the role of the *Agrobacterium* VirF virulence protein inside the host cell, we expressed this protein N-terminally tagged with GFP in *S. cerevisiae* under control of the *MET17* promoter and *CYC1* terminator. As shown in Figure 2A, fluorescence was observed all over the cell, with a somewhat increased concentration in the nucleus. A protein of the expected size of GFP-VirF could be detected by Western blotting using the anti-GFP antibody, indicating that the GFP-VirF fusion protein is expressed in yeast (data not shown). In contrast, a C-terminal GFP fusion did not give detectable fluorescence (data not shown).

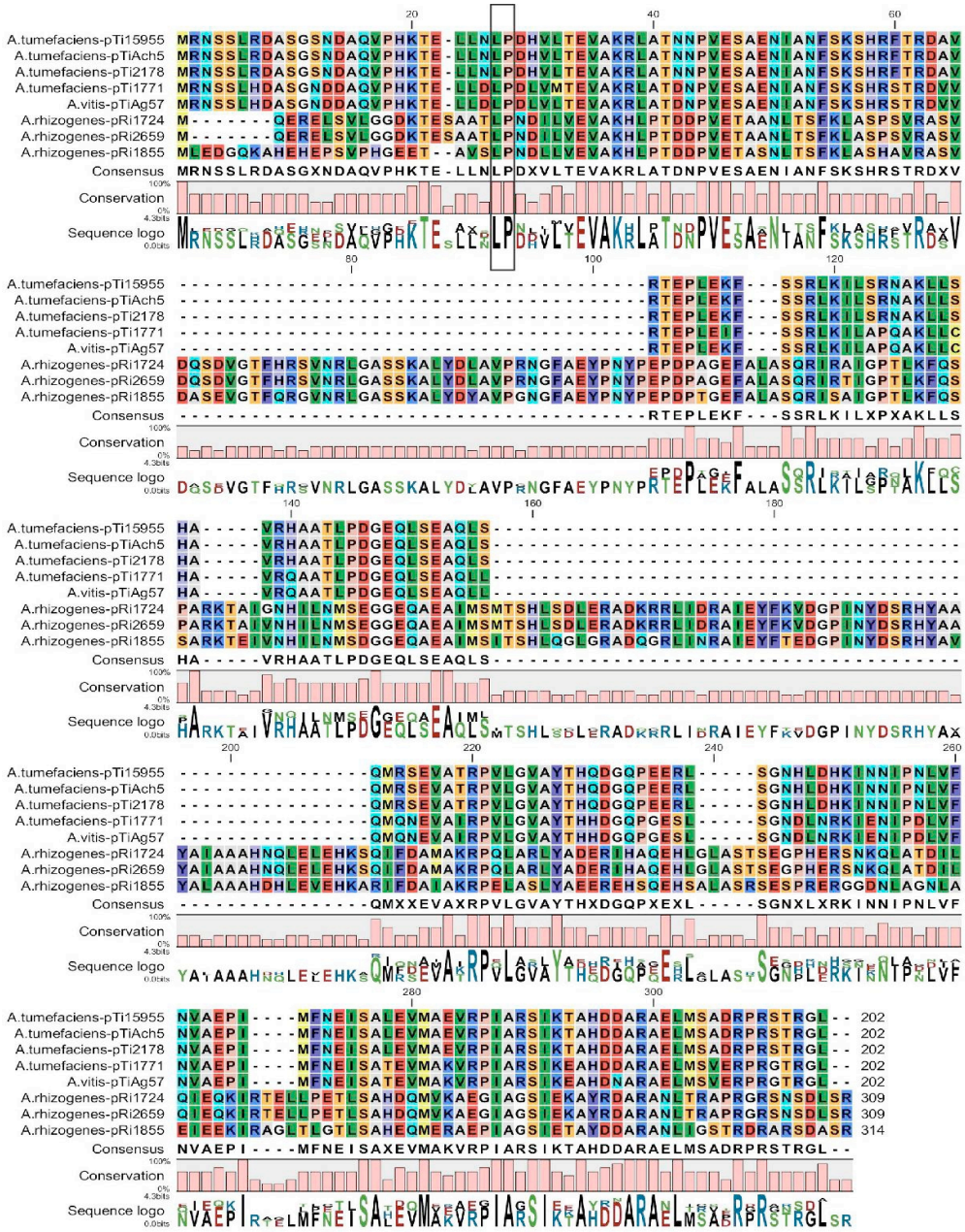

Figure 1 Amino acid sequence alignment of VirF protein from different *Agrobacterium* strains. Box indicates the conserved F-box (LP) motif. The *Agrobacterium* strains are shown at the left side of the aligned amino acid sequence. The plasmids of all *A. tumefaciens* are octopine type Ti plasmids; the plasmid of *A. vitis* is also an octopine type Ti plasmid; the plasmids pRi1724, pRi2659 and pRi1855 of *A. rhizogenes* are mikomopine, cucumopine and agropine type Ri plasmids, respectively. Accession numbers are : NP_059824.1 (pTi15955), AAC97602.1 (pTiAg57), NP_066753.1 (pRi1724) and YP_001961117.1 (pRi2659). The other four *Agrobacterium* strains were sequenced in our group (Hooykaas, unpublished data).

Effect of VirF on the localization and levels of VirE2 and VIP1 in *S. cerevisiae*
According to experiments performed in yeast it has been proposed that VirF forms an SCF complex and that this complex mediates ubiquitination of the host VIP1 and the *Agrobacterium* VirE2 proteins resulting in proteasomal degradation of these proteins allowing integration of the T-DNA into one of the host chromosomes (Tzfira *et al*, 2004). To find out whether VirF indeed plays a role in the degradation of VirE2 and VIP1 in *S. cerevisiae*, we first expressed VirE2 N-terminally fused to YFP and VIP1 N-terminally fused to GFP in *S. cerevisiae*. As shown in Figure 2C, YFP-VirE2 was observed in a filamentous structure which was shown before to colocalize with microtubules (P.A. Sakalis, unpublished observations). GFP-VIP1 was observed exclusively in the yeast nucleus (Figure 2D-F), but not homogenously. This localization differs from that reported for plants (Djamei *et al*, 2007). In plants, VIP1 resides both in the cytoplasm and the nucleus, whereas after phosphorylation by MPK3 in response to the presence of a pathogen, it relocalizes from the cytoplasm into the nucleus.

To investigate the effect of VirF on the expression levels and localization of VirE2 and VIP1, we expressed untagged VirF in cells expressing YFP-VirE2 and CFP-VIP1. As shown in Figure 3A, expression of VirF did not affect the localization of YFP-VirE2 and CFP-VIP1. The fluorescence intensity of CFP-VIP1 and YFP-VirE2 expression did not change after co-expression of VirF (data not shown). To quantify the effect of VirF on the level of VIP1, we expressed GFP-VIP1 in the absence or presence of VirF and in the absence or presence of 3xHA-tagged VirE2 and determined GFP fluorescence in three independent transformants by flow cytometry. The experiment was repeated four times. Our results showed that both in the absence or presence of 3xHA-VirE2, the levels of GFP-VIP1 were not significant affected by expression of VirF (without 3xHAVirE2: GFPVIP1 fluorescence in the presence of VirF normalized to that in the absence of VirF: 1.05±0.06; in the presence of 3xHAVirE2, GFPVIP1 fluorescence in the presence of VirF normalized to that in the absence of VirF: 0.89±0.19). Similarly, we quantified the effect of VirF on the level of VirE2. To this end, we expressed YFP-VirE2 in the absence or presence of VirF in the absence or presence of 3xMyc-tagged VIP1 and determined YFP fluorescence in twelve independent transformants by flow cytometry. Similarly, the levels of YFP-VirE2 were not affected by VirF (without 3xMyc-VIP1: YFP-VirE2 fluorescence in the presence of VirF normalized to that in the absence of VirF: 0.97±0.04; in the presence of 3xMyc-VIP1: YFP-VirE2 fluorescence in the presence of VirF normalized to that in the absence of VirF: 0.94±0.09).

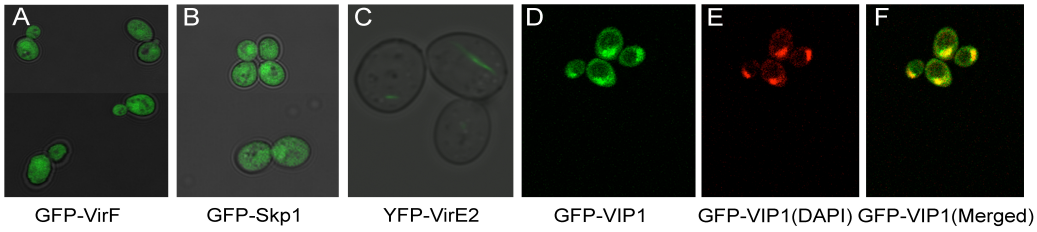

Figure 2 Expression and localization of GFP-VirF, GFP-Skp1, YFP-VirE2 and GFP-VIP1 in *S. cerevisiae*. Confocal microscopy of CEN.PK113-3B with pUG34[virF] (A), CEN.PK113-3B with pUG34[SKP1] (B) GG426-304YFP-*virE2* (C) and CEN.PK113-3B with pUG34[VIP1] (D, E, F) cells.

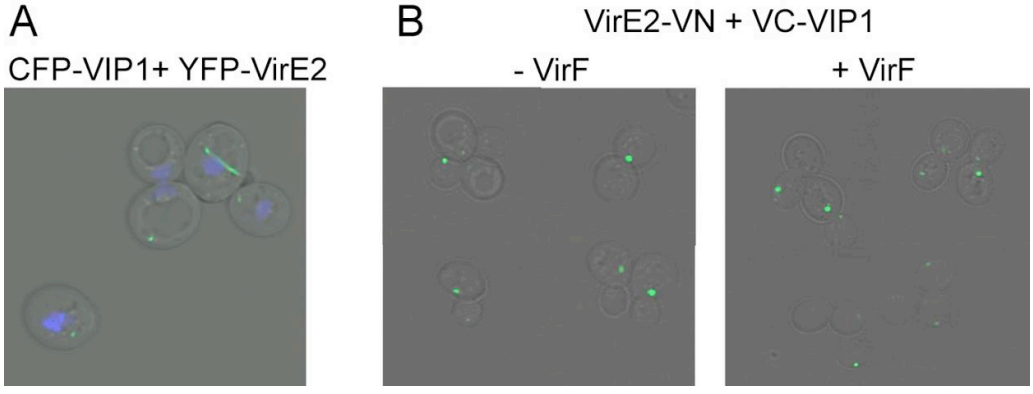

Figure 3. Effect of VirF on VirE2 and VIP1. A, localization of CFP-VIP1 and YFP-VirE2 in the presence of VirF; B, effect of VirF on BiFC of VirE2-VN and VC-VIP1. Confocal microscopy of: A, 426-304YFP-*virE2*/ 435-305CFP-*VIP1* containing pUG34ΔGFP[virF]; B, 426-ΔGFP or 426-virFΔGFP containing pUG35VN[virE2] and pUG34VC[VIP1].

VirF may induce ubiquitination of VirE2 and/or VIP1 when VirE2 and VIP1 are present in a complex. To visualize the VirE2-VIP1 complex, the Bimolecular fluorescence complementation (BiFC) method was used. This approach allows visualization of protein-protein interactions in the living cell and is based on the association of fluorescent protein fragments attached to sequences of two putative interaction partner proteins. The fluorescence signal only appears if the studied proteins physically interact and the two fragments of the fluorescent protein, the YFP analog Venus in our case, come into close proximity to each other (Hu *et al*, 2002; Sung & Huh, 2007). After co-expression of VirE2-VN (C-terminal fusion of N-terminal part of Venus to VirE2, VN) and VC-VIP1 (N-terminal fusion of C-terminal part of Venus to VIP1, VC) clear spots were visible (Figure 3B), indicating that also in yeast VIP1 and VirE2 interact. If VirF induces ubiquitination and degradation of VirE2 and VIP1 when present in a complex, the VirE2-VIP1 BiFC signal is expected to be lower in cells expressing VirF than in control cells.

However, as shown in Figure 3B similar fluorescence was observed in cells with or without VirF. Quantification of the BiFC fluorescence in twelve independent transformants by flow cytometry confirmed the lack of a significant effect of VirF (data not shown).

We next confirmed this observation by an independent approach. To this end, 3×HA- tagged VirE2 and 3×Myc-tagged VIP1 were stably expressed in yeast in the absence and presence of VirF. Figure 4 shows that the amount of 3×HA-tagged VirE2 and 3×Myc-tagged VIP1 were not altered after expression of VirF. Similar results were found when 3×HA-tagged VirE2 and 3×Myc-tagged VIP1 were expressed in the same cell (data not shown). Taken together, these observations do not support a role of VirF in degradation of VirE2 and VIP1 in yeast.

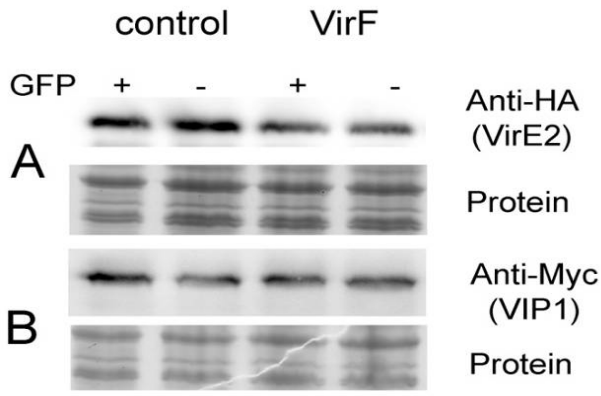

Figure 4. Western blot analysis of the effect of VirF on the levels of VirE2 and VIP1. A. Analysis of 426-304HA-VirE2 cells containing pUG34, pUG34ΔGFP, pUG34[virF] or pUG34ΔGFP[virF] by Western blotting using the anti-HA antibody. B. Analysis of 435-305Myc-VIP1cells containing pUG34, pUG34ΔGFP, pUG34[virF] or pUG34ΔGFP[virF] by Western blotting using the anti-Myc antibody.

Interactions of VirF with VirE2 and VIP1

In our study, we failed to show that VirE2 and VIP1 were degraded by VirF in yeast. For VirF-mediated ubiquitination of VirE2 and VIP1, it is necessary that VirF interacts with these proteins to form an SCF-complex. It has already been reported that VirF binds to VIP1 (Tzfira *et al*, 2004) and through VIP1 also with VirE2 in plant cells. Therefore, we studied whether VirF interacts with VirE2 and/or VIP1 also in yeast. To investigate a possible interaction of VirF with VirE2 and VIP1, the VC-VirF together with VirE2-VN was expressed in yeast. As shown in Figure 5A, cells expressing VC-VirF and VirE2-VN showed a clear fluorescent signal in spots, indicating an interaction between VirF and VirE2. This fluorescence was not observed in cells expressing VC-VirF in combination with free VN or in cells expressing VirE2-VN in combination with free VC (Figure 5A).

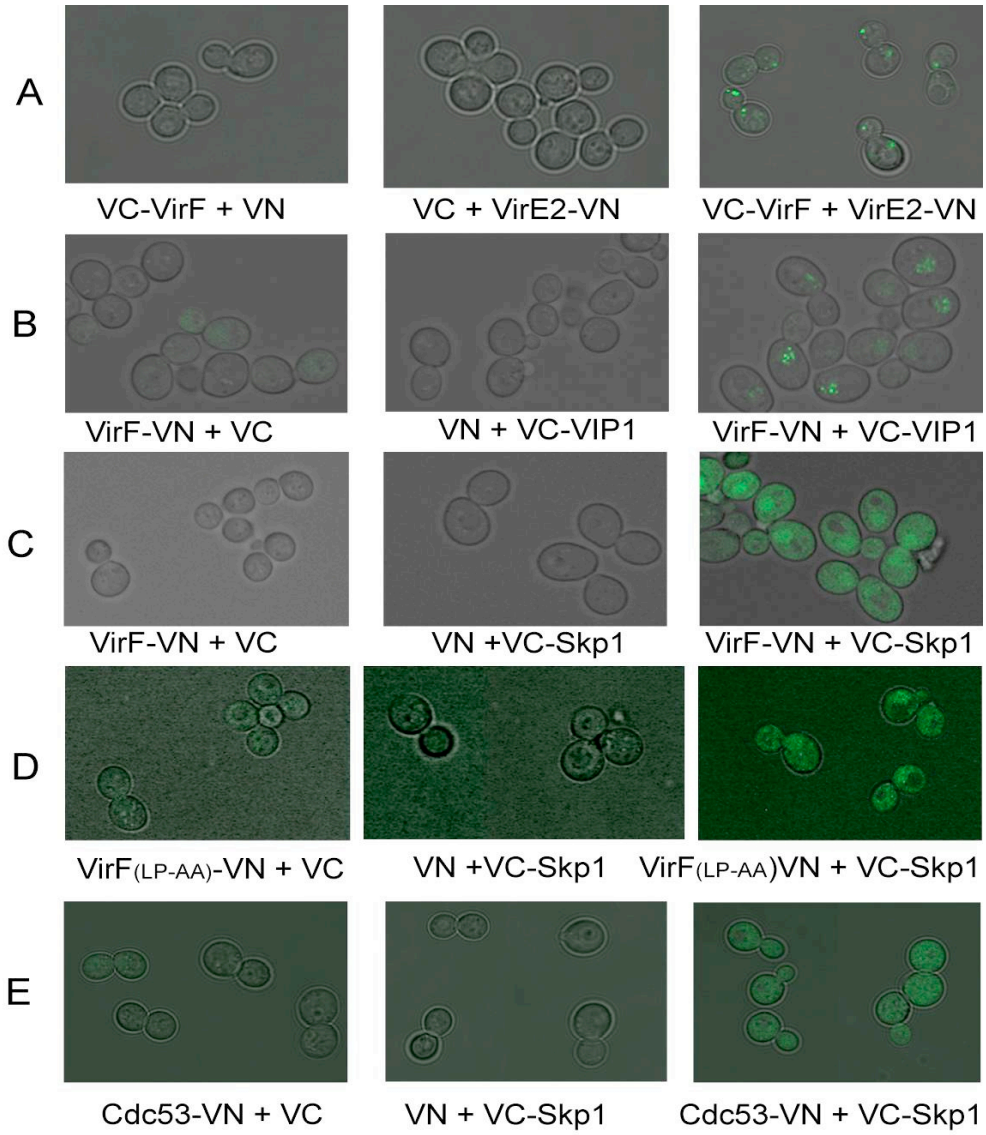

Figure 5 BiFC analysis of the interaction of VirF with VirE2 (A), VIP1 (B) and Skp1 (C) and of Skp1 with Cdc53 (E). Confocal microscopy of: A, CEN.PK113-3B containing the combinations pUG34VC[virF]–pUG35VN, pUG34VC–pUG35VN[virE2] and pUG34VC[virF]–pUG35VN[virE2]; B, CEN.PK113-3B containing the combinations pUG35VN[virF]–pUG34VC, pUG35VN–pUG34VC[VIP1] and pUG35VN[virF]–pUG34VC[VIP1]; C, CEN.PK113-3B containing the combinations pUG35VN[virF]–pUG34VC, pUG35VN–pUG34VC[SKP1] and pUG35VN[virF]–pUG34VC[SKP1]; D, CEN.PK113-3B containing the combinations pUG35VN[virF(LP-AA)]–pUG34VC, pUG35VN–pUG34VC[SKP1] and pUG35VN[virF(LP-AA)]–pUG34VC[SKP1]; E, CEN.PK113-3B containing the combinations pUG35VN[CDC53]–pUG34VC, pUG35VN–pUG34VC[SKP1] and pUG35VN[CDC53]–pUG34VC[SKP1]

To investigate the interaction between VirF and VIP1 we expressed VC-VIP1 together with VirF-VN. As shown in Figure 5B, this co-expression resulted in a clear fluorescence in the nucleus. Expression of VC-VIP1 together with free VN resulted in a very weak fluorescence all over the cell, clearly distinct from that observed after expression of VC-VIP1 together with VirF-VN, thus confirming the interaction between VirF and VIP1. The interaction seen in the BiFC experiments between VirF and VIP1 was in line with previous data obtained from plant cells; however, interaction between VirF and VirE2 was not seen in plant cells. Therefore, in order to verify these findings, we used another biochemical approach to study this, namely *in vitro* pull down assays. To this end, magnetic beads were loaded with 6×His tagged VirF produced in *E. coli* and incubated with protein extracts from yeast cells expressing both YFP-VirE2 and CFP-VIP1. CFP-VIP1 was bound to VirF-loaded beads but not to control beads. On the other hand, YFP-VirE2 was not bound (data not shown). This indicates that the signal seen in the BiFC experiments after co-expression of VC-VirF and VirE2-VN does not represent a genuine stable interaction.

No clear evidence for an F-box mediated association between VirF and Skp1 *in S.cerevisiae*

VirF contains a putative F-box domain by which it can interact with plant homolog of the yeast Skp1 protein, suggesting that VirF can form an SCF-complex and that VirF plays a role in targeted protein degradation (Schrammeijer *et al*, 2001). To study whether VirF also binds to the yeast Skp1 protein, we first studied the localization of GFP-Skp1. As shown in Figure 2B, GFP-Skp1 has a similar localization as GFP-VirF. To study the interaction between VirF and yeast Skp1, initially the BiFC method was used.

Confocal microscopy of cells expressing VirF-VN (C-terminal fusion of N-terminal part of Venus to VirF, VN) and VC-Skp1 (N-terminal fusion of C-terminal part of Venus to VirF, VC) did show a clear fluorescent signal all over the cell, indicating an interaction between VirF and Skp1 (Figure 5C). Similarly, expression of VC-Skp1 together with Cdc53-VN results in a clear fluorescent signal all over the cell (Figure 5E), indicating an interaction between Skp1 and the cullin Cdc53 required for the formation of an SCF complex. The control cells with VirF-VN and free VC or with free VN and VC-Skp1 had hardly detectable fluorescence. This difference in fluorescence was also confirmed by using flow cytometry (data not shown), indicating a specific interaction between VirF-VN and VC-Skp1. We also tried the combination VC-VirF with Skp1-VN. Due to the high background fluorescence of the Skp1-VN fusion, it was difficult to draw conclusions in this case.

To study whether the putative F-box domain of VirF is involved in the binding of VirF to Skp1, we investigated whether the mutated VirF [VirF(LP-AA)] still binds to the Skp1 protein using the BiFC method. As shown in Figure 5D, in

cells expressing VirF (LP-AA)-VN and VC-Skp1, clear fluorescence was visible. Quantification of the BiFC fluorescence in twelve independent transformants by flow cytometry confirmed the lack of effect of mutation of the F-box of VirF on binding to Skp1 (6.56 ± 1.48, for VC-VirF + Skp1-VN; and 6.29 ± 1.48, for VC-VirF(LP-AA) + Skp1-VN; 11.36 ± 2.57, for VirF-VN + VC-Skp1 and 12.66 ± 2.55, for VirF(LP-AA)-VN + VC-Skp1; all in arbitrary units). Thus, the binding of VirF to yeast Skp1 observed by the BiFC method was not dependent on an intact F-box domain, suggesting that VirF does not form a classical SCF-complex in yeast.

In order to verify the BiFC results, an in vitro pull-down method was used. To this end, GST-VirF, GST-VirF (LP-AA) and free GST were expressed in *E. coli* and bound to glutathione-beads. These beads were incubated with 6×His-Skp1 or 6×His-ASK1 (the plant homolog of Skp1) and the formed complexes were eluted with glutathione. As shown in Figure 6, 6×His-ASK1 copurified with GST-VirF whereas 6×His-Skp1 did not. After prolonged exposure of the blot, very weak bands of 6×His-Skp1 were visible in both the lane of GST-VirF and the controls, indicating a background signal (data not shown). Because the F-box domain enables proteins to bind to the Skp1 homologous proteins to form an SCF-complex, the binding of 6×His-ASK1 was greatly reduced after mutation of the VirF F-box domain (Schrammeijer *et al*, 2001).

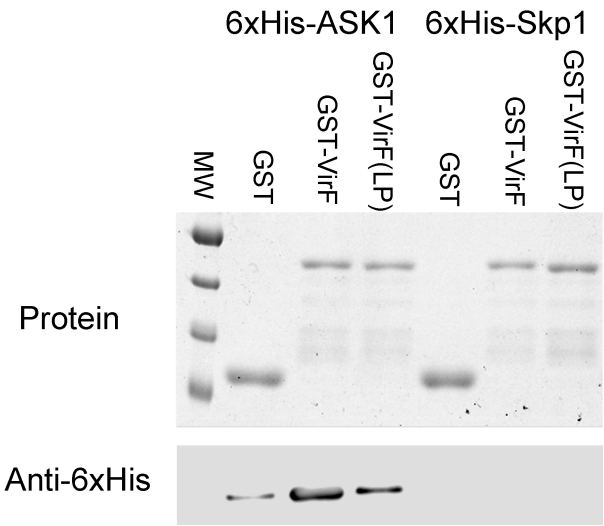

Figure 6. 6×His-ASK1 but not 6×His-Skp1 copurifies with GST-VirF. GST, GST-VirF and GST-VirF (LP) were expressed in *E. coli* and immobilized on GST-agarose beads. Subsequently, the loaded beads were incubated with 6×His-ASK1 or 6×His-Skp1, purified after expression in *E. coli*. Bound proteins were eluted by incubation with glutathione and analyzed by polyacrylamide gel electrophoresis and coomassie staining (protein) to detect the GST-tagged proteins and Western blotting using the anti-6×His antibody to detect 6×His-ASK1 and 6×His-Skp1. MW, molecular weight marker; bands shown: from top to bottom: 55, 40,35 and 25 kDa.

The interaction between VirF and Skp1 was further investigated using the yeast two-hybrid method. By this approach we were again unable to obtain evidence for an interaction between VirF and Skp1, whereas we did find a positive result for the interaction between VirF and ASK1 (data not shown). In another approach to study the interaction of VirF with Skp1 we expressed 6xhistine-tagged Skp1 under control of the inducible *GAL1* promoter in yeast strains 426-305Myc-VirF and 426-305HA-VirF, expressing 3xMyc- and 3xHA-tagged VirF, respectively. Again, we were unable to detect tagged VirF co-purifying with 6xhistine-tagged Skp1 (data not shown). A similar experiment using ASK1 instead of Skp1 was not possible as we were unable to express 6xHis-tagged ASK1 by galactose induction in these strains.

In conclusion, an interaction between VirF and Skp1 was not revealed by yeast two-hybrid analysis and *in vitro* pull-down experiment. The signal seen in BiFC may be due to an irreversible reconstitution of YFP by a transient VirF-Skp1 interaction (Ohad *et al*, 2007).

Altogether we were unable to present evidence for an F-box domain-dependent interaction of VirF with Skp1. Recently, it has been reported that in plants VirF is targeted for degradation through the host ubiquitin-proteasome pathway (Magori & Citovsky, 2011; Zaltsman *et al*, 2013). The interaction between VirF and Skp1 as shown here by the BiFC technique (Fig. 5C) may be an initial step in this process.

As VirF interacts with ASK1, VirF together with ASK1 might form an active SCF-complex in yeast that can mediate degradation of VIP1. To investigate this possibility, we expressed ASK1 under control of the *PGK1* promoter using plasmid pJS95, under control of the *MET17* promoter using plasmid pUG34ΔGFP[ASK1] or under control of the *MET17* promoter as an 3xHA-fusion using plasmid pUG34-HA[ASK1], respectively, in yeast and analyzed the effect of expression of VirF on the levels of GFP-VIP1 by flow cytometry. For quantification, twelve independent transformants were used in each experiment and the experiments were repeated six times. However, in none of the investigated strains expression of VirF resulted in a significant decrease of the levels of GFP-VIP1 (pJS95: GFP-VIP1 fluorescence in the presence of VirF normalized to that in the absence of VirF: 0.90±0.06; pUG34ΔGFP[ASK1]: GFP-VIP1 fluorescence in the presence of VirF normalized to that in the absence of VirF: 0.89± 0.18; pUG34-HA[ASK1]: GFP-VIP1 fluorescence in the presence of VirF normalized to that in the absence of VirF: 0.92 ± 0.14).-Thus also expressing the natural plant Skp1 homolog did not rescue the ability of VirF to target VIP1 for degradation.

Effect of VirF on genome-wide transcription

The VIP1 protein is a transcription factor. No homologs of this transcription

factor are found in *S. cerevisae*. However, in yeast other transcription factors, i.e. transcription factors having little sequence homology to the VIP1 protein, may have a similar function. These putative transcription factors may be degraded in a VirF-dependent way. Degradation of a transcription factor will most likely result in a reduced transcription of the genes of which expression is regulated by this transcription factor. To investigate whether in *S. cerevisiae* VirF can induce degradation of transcription factors we expressed VirF in *S. cerevisiae* and analyzed genome-wide transcription by using DNA microarrays. To this end we expressed the *Agrobacterium virF* gene under control of the yeast *MET17* promoter and *CYC1* terminator in the CEN.PK111-32D strain after integration into the *LEU2* locus yielding strains GG3275. During cultivation of the constructed strains in minimal medium no differences in growth rate could be detected (data not shown). Northern blot analysis showed that in RNA isolated from exponential cultures in minimal medium *virF* mRNA could easily be detected in the relevant strains, indicating correct expression of *virF* at the transcriptional level (data not shown).

To investigate the effect of VirF on genome-wide transcription in *S. cerevisiae*, the strains GG3275 (VirF) and GG3277 (control) were grown in triplicate in SD medium and cells were harvested in the exponential phase. RNA was isolated and analyzed using Affymetrix GeneChip Yeast genome 2.0 microarrays. For each probe on the microarray the average hybridization intensity of the three microarrays of strains GG3275 (VirF) and GG3277 (control) was calculated. Figure 7 shows a comparison of gene expression in strain GG3275 (VirF) with that in GG3277 (control). It is obvious from Figure 7 and Table 4 that the effect of VirF is very minor.

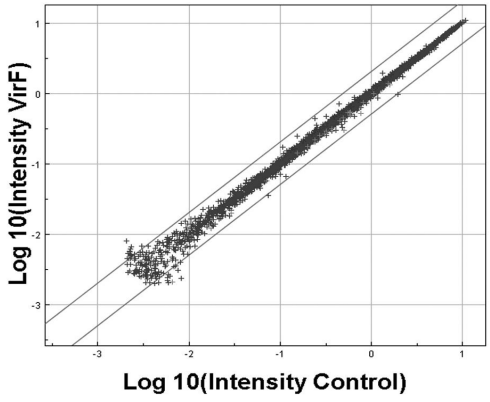

Figure 7. Analysis of the effect of expression of VirF on genome-wide gene expression using the Rosetta program. Lines corresponding to a 2-fold increased or decreased expression are shown.

In Table 4, the genes are listed of which the expression level changed significantly (P<0.01) at least 1.3-fold upon expression of VirF. No genes were upregulated more than two times. The most up-regulated gene (1.6-fold) by expression of VirF is *HSP12* encoding a heat shock protein, whereas the expression of none of the *S. cerevisiae* genes was significantly down-regulated more than 1.3-fold.

Table 4. Genes of which the expression was increased significantly (P<0.01) at least 1.3-fold upon expression of VirF (analyzed using the Bioconductor program)

Gene	Fold change	Probability (P-value t-test)	Function (*Saccharomyces* Genome Database)
HSP12	1.6	0.0099	Plasma membrane protein involved in maintaining membrane organization in stress conditions; induced by heat shock, oxidative stress, osmostress, stationary phase, glucose depletion, oleate and alcohol; regulated by HOG and Ras-PKA pathways
HSP26	1.5	0.0006	Small heat shock protein with chaperone activity; forms hollow, sphere-shaped oligomers that suppress unfolded proteins aggregation; oligomer activation requires heat-induced conformational change; also has mRNA binding activity
DDR2	1.4	0.0001	Multistress response protein, expression is activated by a variety of xenobiotic agents and environmental or physiological stresses
GPH1	1.4	0.0015	Non-essential glycogen phosphorylase required for the mobilization of glycogen, activity is regulated by cyclic AMP-mediated phosphorylation, expression is regulated by stress-response elements and by the HOG MAP kinase pathway.

Discussion

Analysis of the *Agrobacterium* VirF amino acid sequence revealed the presence of a putative F-box domain (Schrammeijer *et al*, 2001), and a T4SS translocation signal at the C-terminus (Vergunst *et al*, 2000). The F-box domain mediates protein–protein interactions and it interacts directly with Skp1 orthologs, a component of the Skp1–Cullin–F-box complex (or SCF complex). The F box proteins act as receptors, which attract specific proteins to the SCF complex for ubiquitination and subsequent proteolysis. It has been shown before that VirF binds to the ASK1 protein, one of the *Arabidopsis* orthologs of the yeast Skp1 protein, suggesting that in *planta* VirF indeed forms an SCF-complex and that VirF is involved in targeted proteolysis (Schrammeijer *et al*, 2001). In this study, we were unable to provide evidence that VirF bound to *S. cerevisiae* Skp1 in an

F-box domain dependent way, suggesting that in yeast VirF may not form a SCF-complex. Only using the BiFC method a clear fluorescent signal was observed after co-expression of VirF-VN and VC-Skp1 (Fig. 5C). However, mutation of the VirF F-box domain did not affect this interaction. Using co-purification experiments we showed an interaction between GST-VirF and 6×His-ASK1, but not between GST-VirF and 6×His-Skp1 (Fig. 6). Furthermore, yeast two-hybrid analysis did not give evidence for an interaction between VirF and Skp1, whereas it did give evidence for an interaction between VirF and ASK1 (data not shown). Besides, we also failed to detect an interaction between VirF and Skp1 after expression in yeast by co-purification methodology.

Upon *Agrobacterium*-mediated transformation of both plants and yeast, VirF is translocated into the host cell (Vergunst *et al*, 2000; Schrammeijer *et al*, 2003). In plants VirF binds to VIP1, a transcription factor that regulates the expression of the PR1 pathogenesis-related gene (Tzfira *et al*, 2004; Djamei *et al*, 2007). VIP1 also binds to the *Agrobacterium* VirE2 protein (Tzfira *et al*, 2001). According to a current hypothesis, VirF destabilizes VIP1 and VirE2 resulting in uncoating of the T-DNA enabling integration of the T-DNA into one of the host chromosomes (Tzfira *et al*, 2004). In contrast to reported results (Tzfira *et al*, 2004) we did not obtain any evidence for a role of VirF in the destabilization of the VirE2 and VIP1 proteins in yeast. At the moment we do not know how these differences can be explained. Part of the evidence that VirF in yeast is involved in this process was based on experiments in which expression of GFP-tagged VIP1, VirE2 and VirD2 was controlled by a galactose inducible promoter. During the transfer from a medium with galactose to that with glucose to turn-off the promoter not only the activity of the inducible promoter may have been affected but also many other cellular processes, making it difficult to draw conclusions.

The VIP1 protein is a plant-specific transcription factor and *S. cerevisiae* lacks transcription factors homologous to VIP1. However, transcription factors with low homology to VIP1 may have a similar function in *S. cerevisae*. In this study we addressed the question whether VirF is able to stimulate the degradation of transcription factors in *S. cerevisiae* by analyzing the effect of VirF expression on genome-wide transcription. As shown in Figure 7 and Table 4, the effect of VirF expression is very minor. Altogether, this study does not provide evidence for VirF-mediated degradation of transcription factors and/or their regulators in yeast.

The results of this study are in line with a less important role of VirF during the *Agrobacterium*-mediated transformation of *S. cerevisiae*. This is further supported by the observation that *virF* is not required for the transformation of *S. cerevisiae* (Bundock *et al*, 1995). On the other hand, VirF mediated ubiquitination and proteasomal degradation of proteins associated with the

T-complex may be imperative during the transformation of plants (Anand et al, 2012). This is in line with the observations that VirF binds in an F-box dependent way to the plant ASK1 protein (Schrammeijer *et al*, 2001) and that octopine and nopaline strains of *Agrobacterium* differ in their virulence on *Nicotiana glauca*, due to the absence of a functional *virF* locus in nopaline strains (Melchers et al., 1990). The targets of such VirF containing plant SCF complexes may include plant specific proteins involved in defense or otherwise inhibitory to transformation, as identified previously by Jurado (2011). If T-complexes are formed in vivo, decoating may occur not only by targeted proteolysis, but also by the action of helicases or other DNA recombination related proteins.

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References

Abràmoff MD, Magalhães PJ & Ram SJ (2004) Image Processing with ImageJ. *Biophotonics international* **11:** 36-42

Anand A, Krichevsky A, Schornack S, Lahaye T, Tzfira T, Tang Y, Citovsky V & Mysore KS (2007) Arabidopsis VirE2 Interacting protein2 is required for Agrobacterium T-DNA integration in plants. *Plant Cell* **19:** 1695-1708

Anand A, Rojas CM, Tang Y & Mysore KS (2012) Several components of SKP1 / Cullin / F-box E3 ubiquitin ligase complex and associated factors play a role in Agrobacterium-mediated plant transformation. *New Phytologist* **195:** 203-216

van Attikum H, Bundock P & Hooykaas PJ (2001) Non-homologous end-joining proteins are required for Agrobacterium T-DNA integration. *EMBO J* **20:** 6550-6558

van Attikum H & Hooykaas PJ (2003) Genetic requirements for the targeted integration of Agrobacterium T-DNA in Saccharomyces cerevisiae. *Nucleic Acids Res* **31:** 826-832

Bundock P, den Dulk-Ras A, Beijersbergen A & Hooykaas PJ (1995) Trans-kingdom T-DNA transfer from Agrobacterium tumefaciens to Saccharomyces cerevisiae. *EMBO J* **14:** 3206-3214

Djamei A, Pitzschke A, Nakagami H, Rajh I & Hirt H (2007) Trojan horse strategy in Agrobacterium transformation: abusing MAPK defense signaling. *Science (80-)* **318:** 453-456

García-Rodríguez, Fernando M, Schrammeijer B & Hooykaas, P, J J. (2006) The Agrobacterium VirE3 effector protein: a potential plant transcriptional activator. *Nucleic Acids Res* **34:** 6496-504

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, *et al* (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* **5:** R80

Gietz RD, Schiestl RH, Willems AR & Woods RA (1995) Studies on the transformation of intact

yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* **11:** 355-360
Gietz RD & Sugino A (1988) New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction. *Gene* **74:** 527-534
Guan KL, Dixon JE (1991) Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal Biochem*. **192**:262–267
van Hemert MJ, Deelder AM, Molenaar C, Steensma HY & van Heusden GPH (2003) Self-association of the spindle pole body-related intermediate filament protein Fin1p and its phosphorylation-dependent interaction with 14-3-3 proteins in yeast. *J Biol Chem* **278:** 15049-15055
Hu C-deng, Chinenov Y, Kerppola TK, Hughes H & Arbor A (2002) Visualization of Interactions among bZIP and Rel Family Proteins in Living Cells Using Bimolecular Fluorescence Complementation. **9:** 789-798
Lacroix B, Loyter A & Citovsky V (2008) Association of the Agrobacterium T-DNA-protein complex with plant nucleosomes. *Proc Natl Acad Sci U S A* **105:** 15429-15434
Magori S & Citovsky V (2011) Agrobacterium counteracts host-induced degradation of its effector F-box protein. *Sci Signal* **4:** 1-7
Ohad N, Shichrur K & Yalovsky S (2007) The analysis of protein-protein interactions in plants by bimolecular fluorescence complementation. *Plant Physiol* **145:** 1090-1099
Piers KL, Heath JD, Liang X, Stephens KM & Nester EW (1996) Agrobacterium tumefaciens-mediated transformation of yeast. *Proc Natl Acad Sci U S A* **93:** 1613-1618
Schouten J, Kam RJD & Fetter K (2000) Overexpression of Arabidopsis thaliana SKP1 homologues in yeast inactivates the Mig1 repressor by destabilising the F-box protein Grr1. *Mol Gen Genet* **34:** 309-319
Schrammeijer B, den Dulk-Ras A, Vergunst, A.C., Jurado Jacome E & Hooykaas PJJ (2003) Analysis of Vir protein translocation from Agrobacterium tumefaciens using Saccharomyces cerevisiae as a model: evidence for transport of a novel effector protein VirE3. *Nucleic Acids Res* **31:** 860-868
Schrammeijer B, Risseeuw E, Pansegrau W, Regensburg-Tuink, T. J., Crosby,W. L. & Hooykaas PJJ (2001) Interaction of the virulence protein VirF of Agrobacterium tumefaciens with plant homologs of the yeast Skp1 protein. *Curr Biol* **11:** 258-262
Soltani J, van Heusden GPH & Hooykaas PJJ (2008) Agrobacterium-mediated transformation of non-plant organisms. In *Agrobacterium: From Biology to Biotechnology*, Tzfira T & Citovsky V (eds) pp 649 - 675. Springer New York
Sung M-kyung & Huh W-ki (2007) Bimolecular fluorescence complementation analysis system for in vivo detection of protein – protein interaction in Saccharomyces cerevisiae. *Yeast* **24:** 767-775
Tzfira T, Vaidya M & Citovsky V (2001) VIP1, an Arabidopsis protein that interacts with Agrobacterium VirE2, is involved in VirE2 nuclear import and Agrobacterium infectivity. *EMBO J* **20:** 3596-3607
Tzfira T, Vaidya M & Citovsky V (2004) Involvement of targeted proteolysis in plant genetic transformation by Agrobacterium. *Nature* **431:** 87-92
Vergunst A., Schrammeijer B, den Dulk-Ras A, de Vlaam CM., Regensburg-Tuink TJ. & Hooykaas PJJ (2000) VirB/D4-dependent protein translocation from Agrobacterium into plant cells. *Science* **290:** 979-982
Zaltsman A, Lacroix B, Gafni Y & Citovsky V (2013) Disassembly of synthetic Agrobacterium T-DNA-protein complexes via the host SCFVBF ubiquitin-ligase complex pathway. *Proc Natl Acad Sci U S A* **110:** 169-174
Zonneveld BJM (1986) Cheap and simple yeast media. *J Microbiol Methods* **4:** 287-291

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