

Functional analysis of agrobacterium virulence genes Niu, X.

Citation

Niu, X. (2013, June 25). *Functional analysis of agrobacterium virulence genes*. Retrieved from https://hdl.handle.net/1887/21014

Version:	Corrected Publisher's Version			
License:	<u>Licence agreement concerning inclusion of doctoral thesis in the</u> <u>Institutional Repository of the University of Leiden</u>			
Downloaded from:	https://hdl.handle.net/1887/21014			

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <u>http://hdl.handle.net/1887/21014</u> holds various files of this Leiden University dissertation.

Author: Niu, Xiaolei Title: Functional analysis of agrobacterium virulence genes Issue Date: 2013-06-25

Chapter 2

Transformation of yeast F-box deletion strains by *Agrobacterium* strains deficient in VirF

Xiaolei Niu, G.Paul H. van Heusden and Paul J.J. Hooykaas Department of Molecular and Developmental Genetics, Institute of Biology, Leiden University, Sylviusweg 72, 2333BE Leiden, the Netherland

Abstract

The gram-negative soil bacterium Agrobacterium tumefaciens can cause crown gall tumors in plants by transferring a piece of oncogenic DNA, called T-DNA, into plant cells. The virulence genes located in the vir region of the Ti plasmid play an important role in the transformation process. A number of the encoded virulence proteins are also translocated into plant cells during transformation. One of these proteins, VirF, is a putative prokaryotic F-box protein. F-box proteins are components of eukaryotic SCF (Skp1-Cdc53-F box protein) ubiquitin-ligase (E3) complexes, which play a role in ubiquitin-mediated targeted protein degradation. Agrobacterium can also transform yeast and fungi. For the transformation of yeast by Agrobacterium, the VirF protein is not required. However, endogenous yeast F-box proteins may take over the function of VirF during the transformation process. In order to address this hypothesis, yeast strains with deletions of genes encoding each of the yeast F-box proteins were transformed with Agrobacterium wild type and VirF-deficient strains. These studies showed that all 16 viable yeast F-box deletion strains can be transformed equally well by both Agrobacterium wild type and VirF-deficient strains. This indicates that either VirF is not necessary for the transformation of yeast or that its function is substituted by one of the yeast essential F-box proteins.

Introduction

The gram-negative soil bacterium *Agrobacterium tumefaciens* can cause crown gall tumors in plants by transferring part of its tumor-inducing plasmid, the T-DNA, to plant cells, which is followed by integration of the T-DNA into one of the plant chromosomes. The T-DNA contains genes coding for enzymes producing opines and genes responsible for uncontrolled proliferation (Gelvin, 2000, 2003; Hooykaas and Beijersbergen, 1994; Tzfira and Citovsky, 2000; Zhu *et al.*, 2000; Zupan *et al.*, 2000}{Formatting Citation}{Formatting Citation}. Under laboratory conditions *Agrobacterium* T-DNA transfer is not restricted to plants; *Agrobacterium* is also able to transform non-plant hosts such as algae (Kumar *et al.*, 2004), yeasts (Bundock *et al.*, 1995) and filamentous fungi (Gouka *et al.*, 1999; de Groot *et al.*, 1998).

The virulence genes located in the *vir* region of the Ti plasmid, play an important role in the transformation process. The *vir* region encodes proteins involved in T-DNA production and delivery. The transformation process starts with the attachment of *Agrobacterium* to host cells and the sensing by *Agrobacterium*

of plant phenolic signals that are produced by wounded plant cells such as acetosyringone (AS) by the VirA chemoreceptor. This leads to expression of the other *vir* genes. A single stranded copy of the T-region, called the T-strand is generated with the VirD2 protein covalently attached to its 5'-end and T-strand. The T-strand is exported into the host cell through a VirB/D4 type IV secretion system (T4SS). Some Vir proteins including VirE2, VirE3, VirF and VirD5 are effector proteins which are also introduced into host cells by the T4SS (Vergunst *et al.*, 2000). After the T-strand enters the host cytoplasm, it is coated with numerous VirE2 proteins generating the mature T-complex. The T-complex is then targeted into the nucleus by the nuclear localization sequence (NLS) of VirD2 and assisted by the VIP1 protein which also has an NLS and which binds to the T-complex through an interaction with VirE2 (Djamei *et al.*, 2007; Tzfira *et al.*, 2001; Ward *et al.*, 2002). Inside the nucleus, the T-complex is thought to be uncoated, enabling double strand formation and integration into the host genome.

The virF gene was discovered by studying the virulence of different Agrobacterium tumefaciens mutants (Hooykaas et al., 1984). The virF mutant shows decreased virulence on tomato and *Nicotiana glauca*. Nopaline strains, which naturally lack virF, are less virulent than octopine strains on N. glauca (Melchers et al., 1990). Nopaline strains and octopine virF mutants can be complemented for tumorigenicity by coinfection with a helper strain lacking the T-region, but containing VirF (Otten et al., 1985; Regensburg-Tuink and Hooykaas, 1993). Complementation also occurred when VirF is expressed in the host plant, which indicated that VirF may be a translocated effector protein (Regensburg-Tuink and Hooykaas, 1993). This turned out to be the case indeed when tested using the CRAFT assay (Vergunst et al., 2000). The virF gene encodes a putative F-box protein which can interact, through its F-box motif, with the Arabidopsis thaliana ASK1 protein, a plant homologue of the yeast Skp1 protein (Schrammeijer et al., 2001). Both F-box proteins and Skp1 proteins are components of SCF (Skp1-Cullin-F box protein) ubiquitin-ligase (E3) complexes, which play a role in ubiquitinmediated targeted protein degradation (Cardozo and Pagano, 2004; Hua and Vierstra, 2011; Nakayama and Nakayama, 2006; Vierstra, 2009; Welchman et al., 2005). It has been reported that VirF can mediate the degradation of VirE2 and VIP1, and thus it has been proposed that this activity may be responsible for the uncoating of the T-complex (Tzfira et al., 2004). However, also other potential substrates of VirF have been identified (Jurado, 2011).

VirF is not required for the transformation of *Arabidopsis* and many other plant species (Jarchow *et al.*, 1991; Melchers *et al.*, 1990) nor for the transformation of the yeast *Saccharomyces cerevisiae* (Bundock *et al.*, 1995) or fungi (Michielse *et al.*, 2005). Recently, it was published that expression of VBF, an *Arabidopsis* gene encoding an F-box protein, was induced by *Agrobacterium* infection and

that the VBF protein can mimic the function of VirF, thus explaining why VirF is not essential for the transformation of *Arabidopsis* (Zaltsman *et al.*, 2010). In this study, we were interested to see whether similarly, a yeast F-box protein could take over the function of VirF during AMT. To this end, the transformation efficiency of yeast diploid F-box deletion strains in the BY4743 background by *Agrobacterium* wild type and a VirF-deficient mutant was investigated.

Materials and Methods

Yeast strains and media

S. cerevisiae BY4743 (MATa/MATa his3 Δ 1/his3 Δ 1 leu2 Δ 0/leu2 Δ 0 lys2 Δ 0/LYS2 MET15/met15 Δ 0 ura3 Δ 0/ura3 Δ 0) and the collection of homozygous diploid F-box deletion strains in the BY4743 background were obtained from Euroscarf (Frankfurt, Germany). In this study 16 deletion strains deficient in genes encoding (putative) F-box proteins were used (Table 1). All yeast cells were grown at 30 °C in yeast-peptone-dextrose (YPD) liquid medium.

	Gene name	Systematic name
1	DAS1	YJL149W
2	DIA2	YOR080W
3	ELA1	YNL230C
4	GRR1	YJR090C
5	HRT3	YLR097C
6	MDM30	YLR368W
7	MFB1	YDR219C
8	RCY1	YJL204C
9	ROY1	YMR258C
10	SAF1	YBR280C
11	SKP2	YNL311C
12	UFO1	YML088W
13	YDR131C	YDR131C
14	YDR306C	YDR306C
15	YLR224W	YLR224W
16	VIR352W/	VIR352W/

Table 1. Deletion	n strains	lacking	F-box	proteins	used in	this	study.
	i strumb	i u civing	1 000	proteins	uscu m	ting	study.

Note: CDC4 (*YFL009W*) and *MET30* (*YIL046W*) are essential F-box protein genes and were not included in this study.

http://www.yeastgenome.org/cgi-bin/search/luceneQS.fpl?query=F-box

Agrobacterium strains and plasmids

A. tumefaciens strain LBA1100 (Beijersbergen *et al.*, 1992), containing an octopine type helper plasmid with a complete virulence (vir) region but lacking the T-region, was used for yeast transformation experiments. LBA2561, a derivative of LBA1100 with a deletion of virF (Schrammeijer et al., 1998), and LBA3557, a LBA1100 derivative with a triple deletion of virF, virE3 and virD5 (denDulk-Ras and Hooykaas, unpublished) were also used as donors. Those three *Agrobacterium* strains were transformed by electroporation with the binary vectors pRAL7100 and pRAL7101 (Bundock *et al*, 1995). The T-region of plasmid pRAL7100 contains the *URA3* gene flanked by yeast *PDA1* sequences allowing integration into the *PDA1* locus via homologous recombination. The

T-region of plasmid pRAL7101 contains the URA3 gene and the yeast 2μ origin of replication, and after transformation, the T-DNA of this plasmid will circularize and be maintained in yeast as an extra-chromosomal plasmid.

Agrobacterium mediated transformation

Agrobacterium-mediated transformation of yeast was done according to Bundock et al., (1995). Briefly, Agrobacterium strains harboring either the binary vector pRAL7100 or pRAL7101 were grown overnight at 30°C in 20 ml Luria-Bertani medium with kanamycine (40 $\mu g/\mu l$). All yeast recipient strains were grown overnight at 30°C in 20 ml YPD liquid medium. The following day, 1.2 ml of overnight Agrobacterium culture was added to 20 ml of Induction medium supplemented with acetosyringone (AS), and 2 ml of overnight yeast culture was added to 18 ml fresh YPD liquid medium, and both cultures were shaken at 30°C. To optimize the protocol, varying amounts of overnight culture were added to the fresh medium for further subculture. After 6 hours, 1 ml of yeast culture was centrifuged and the cells were resuspended in 0.5 ml Induction medium (IM) and centrifuged again. Yeast cells were resuspended in 1 ml of IM medium without glucose. Then, 60 µl of Agrobacterium culture and 60 µl yeast suspension were added to a 1.5ml Eppendorf tube and vortexed gently. Finally 100 µl of the mixture was pipetted onto a piece of nitrocellulose filter (0.45 mm) placed on an IM plate with AS. The plate was incubated at 22°C for 7 days. Then, the nitrocellulose membrane was transferred to a 2 ml Eppendorf tube, 1 ml MilliQ water was added, and the cells were vortexed for 1 min. Aliguots of 50 μl and 250 μl were applied on MY plates (Zonneveld, 1986) supplemented with cefotaxim (200µg/ml), leucine (3 mg/ml) and histidine (2 mg/ml) to quantify transformants. The total number of survived S. cerevisiae cells was quantified by plating 200 μ l aliquots of 10⁻⁵ and 10⁻⁶ dilutions of the resuspended cells on MY plates containing cefotaxim, leucine, histidine and uracil (2 mg/ml). The efficiency of transformation is indicated as the number of transformed yeast cells divided by the number of survived yeast cells.

Results

Optimization of the protocol for Agrobacterium mediated transformation of yeast

To analyze Agrobacterium-mediated transformation (AMT) of yeast deletion mutants lacking F-box proteins, an efficient transformation protocol is required. Therefore, we first investigated whether the AMT protocol can be optimized for yeast strain BY4743. To this end, the amount of Agrobacterium and yeast cells used for co-cultivation was varied. Different amounts of an overnight culture of Agrobacterium strain LBA1100 harboring either plasmid pRAL7100 or plasmid pRAL7101 and different amounts of an overnight culture of yeast strain BY4743 were used for preparing cultures for co-cultivation (see Materials and

Methods). The T-DNA of plasmid pRAL7100 contains the *URA3* gene flanked by yeast *PDA1* sequences allowing integration into the *PDA1* locus via homologous recombination. The T-DNA of plasmid pRAL7101 contains the *URA3* gene and the yeast 2μ origin of replication, and after transformation, the T-DNA part of this plasmid will circularize and be maintained in yeast as an extra-chromosomal plasmid. As shown in Figure 1, only when the lowest amounts of yeast cells were used, high transformation efficiencies were obtained, irrespective of the amount of *Agrobacterium* culture. Therefore, at the start of subculture, the amount of *Agrobacterium* culture was 5.6 ml and of yeast 0.25 ml.



Figure 1. The effect of varying the amount of *Agrobacterium* and yeast cells on AMT efficiency. The indicated amounts of an overnight culture of *Agrobacterium* LBA1100 containing either plasmid pRAL7100 or plasmid pRAL7101 and of yeast strain BY4743 were used to prepare subcultures for co-cultivation. Agro: *Agrobacterium* culture.

Even lower amounts of overnight yeast cells and higher amounts of overnight *Agrobacterium* cells were used in a second experiment. As shown in Figure 2, transformation efficiencies varied substantially in the different co-cultivations. Nevertheless, the highest transformation efficiencies were obtained when 15 ml of *Agrobacterium* overnight culture was used in combination with 0.05 to 0.25 ml of a yeast overnight culture for subculture.

In order to analyze the transformation of yeast by *Agrobacterium* strains lacking *virF* or lacking *virF* together with *virE3* and *virD5*, we made use of the *Agrobacterium virF* deletion strain LBA2561 and the *virF virE3 virD5* triple mutant strain LBA3557. The mutant strains carrying plasmid pRAL7100 were able to transform yeast strain BY4743, equally well as the wild type under the optimal conditions, confirming that *virF* is not required for the transformation of *S. cerevisiae* (Schrammeijer, 2003). Similar results were obtained for the mutant strains carrying plasmid pRAL7101 (data not shown). The highest transformation efficiency was obtained when using 15 ml of *Agrobacterium* overnight culture in combination with 0.05 ml of a yeast overnight culture for subculture. After six

hours growing in induction medium supplemented with AS for Agrobacterium and liquid YPD medium for yeast, the OD of Agrobacterium strains were always around 1.2 and the OD of yeast strains grown in liquid YPD medium were always around 0.16. The corresponding amounts of Agrobacterium and yeast strains were 5.6×10^9 per ml and 4.2×10^6 per ml, respectively. We used similar amounts of Agrobacterium and yeast for subsequent analysis of AMT of the yeast deletion strains.



Figure 2. The effect of increased amounts of *Agrobacterium* and decreased amounts of yeast cells on AMT efficiency. The indicated amounts of an overnight culture of *Agrobacterium* LBA1100 containing either pRAL7100 or pRAL7101 and of yeast strain BY4743 were used to prepare subcultures for co-cultivation. Solid lines: transformation efficiencies for co-cultivations with *Agrobacterium* carrying pRAL7100; Dashed lines: transformation efficiencies for co-cultivations with *Agrobacterium* carrying pRAL7101. Agro: *Agrobacterium* culture.

AMT of yeast F-box protein deletion strains

In order to investigate whether specific yeast F-box proteins can take over the role of Agrobacterium VirF in the transformation process, we studied the transformation of yeast strains lacking genes encoding F-box proteins by Agrobacterium strains deficient in VirF. Because of the high fluctuation in the transformation efficiencies observed in the different experiments, it was very hard to accurately quantify transformation efficiencies for the various combinations of Agrobacterium and yeast strains. However, qualitative determinations were possible.

S. cerevisiae has sixteen non-essential genes encoding F-box proteins (Table 1). In a single experiment maximally four strains can be analyzed for transformation efficiencies in a convenient way. Therefore, the sixteen F-box protein deletion strains were analyzed in six separate experiments and the transformation

efficiency was determined relative to that of BY4743 transformed in the same experiment. As shown in Table 2, all sixteen F-box protein deletion strains can be transformed by *Agrobacterium* strains lacking *virF* (LBA2561), lacking *virF*, *virE3* and *virD5* (LBA3557) and the control strain LBA1100 irrespective of whether plasmid pRAL7100 or pRAL7101 were presented as T-DNA donor.

Table 2. Transformation efficiencies of F-box protein deletion strains by *Agrobacterium* strains lacking *virF* (LBA2561), lacking *virF*, *virE3* and *virD5* (LBA3557) and the control strain LBA1100. Transformation efficiencies (percentage) were calculated relative to that of BY4743 transformed by LBA1100 in the same experiment. Data shown are the average of the data obtained from two separate filters.

pRAL7100			pRAL7101					
Strains	LBA1100	LBA2561	LBA3557	LBA1100	LBA2561	LBA3557		
Experiment 1								
BY4743	100	105	16	100	106	75		
YLR224W	115	97	20	223	105	29		
RCY1	292	321	22	479	104	119		
MDM30	361	397	152	520	281	101		
Experiment 2	2							
BY4743	100	56	10	100	89	38		
ELA1	93	120	47	683	458	48		
YDR306C	64	88	14	130	204	17		
Experiment 3	3	,	,					
<u>BY4743</u>	100	173	50	100	93	17		
DIA2	32194	2981	2357	3503	3492	906		
GRR1	17162	10701	5095	7641	7228	2921		
SAF1	81	126	43	152	86	24		
Experiment 4	1		,					
<u>BY4743</u>	100	116	62	100	421	143		
HRT3	157	108	54	104	193	235		
UFO1	116	104	35	243	330	103		
YMR258C	103	92	20	202	215	122		
Experiment 5								
BY4743	100	89	26	100	282	49		
SKP2	175	113	42	295	189	64		
YDR131C	136	118	41	240	245	89		
Experiment 6								
BY4743	100	121	46	100	337	130		
YLR352W	30	76	28	84	434	45		
DAS1	99	101	81	347	612	94		
MFB1	102	117	62	150	235	88		

Interestingly, the transformation efficiency of the *DIA2* and *GRR1* deletion mutants was more than ten times higher than that of the other strains. However, this may be explained by the observation that these deletion strains are growing much slower than the other strains and that survival of these strains during the transformation process is much lower, making calculation of the transformation efficiencies cumbersome.

The control strain BY4743 has been transformed in six independent experiments with the three *Agrobacterium* strains. In most experiments the transformation efficiencies observed for the triple deletion strain LBA3557 were somewhat lower than that for the other *Agrobacterium* strains (LBA1100 and LBA2561). However, because of the huge fluctuation in transformation efficiencies, the significance of this observation still has to be established.

Discussion

The Agrobacterium VirF virulence protein plays an important role in the transformation of several plant species like tomato and *N. glauca* (Melchers *et al.*, 1990). However, VirF is not required for the transformation of other plant species like *A. thaliana* or for the transformation of the yeast *S. cerevisiae* (Bundock *et al.*, 1995). This difference in host susceptibility may be caused by presence of host F-box proteins that fulfill similar functions as the Agrobacterium VirF protein in hosts susceptible for transformation by Agrobacterium strains deficient in VirF. Recently, it was found that an Arabidopsis F-box protein named VBF was induced by Agrobacterium infection and VBF can mimic the function of VirF, thus explaining why VirF is not essential for the transformation of *Arabidopsis* (Zaltsman *et al.*, 2010).

In this study, we were interested to see whether a yeast F-box protein could similarly take over the function of VirF. To this end, yeast diploid homozygous deletion strains lacking F-box proteins in the BY4743 background were co-cultivated with *Agrobacterium* wild type and VirF-deficient strains. The yeast *S. cerevisiae* has 18 genes encoding proteins with a (putative) F-box domain, of which two are essential (Saccharomyces genome database, www.yeastgenome. org) (for review see: (Jonkers and Rep, 2009)). As shown in Table 2, all 16 viable F-box protein deletion strains can be transformed by *Agrobacterium* strains deficient in *virF*. This observation indicates either that for the transformation of *S. cerevisiae* the function of VirF is not required or that one of the two essential yeast F-box proteins can substitute for the function of VirF or that two or more yeast F-box proteins have redundant functions in the transformation process.

Acknowledgements

This study was supported by the Chinese Scholarship Council (CSC) and by the division Chemical Sciences (CW) of the Netherlands Organisation of Research (NWO), grant number 700.56.303. We would like to thank Jalal Soltani, Amke den Dulk-Ras and Raymond Brandt for their technical support.

Reference

Beijersbergen, A., Dulk-Ras, A. D., Schilperoort, R. A. and Hooykaas, P. J. (1992). Conjugative transfer by the virulence system of Agrobacterium tumefaciens. *Science* **256**, 1324-1327.

Bundock, P. and Hooykaas, P. J. (1996). Integration of Agrobacterium tumefaciens T-DNA in the Saccharomyces cerevisiae genome by illegitimate recombination. *Proc Natl Acad Sci U S A* **93**, 15272-15275.

Bundock, P., den Dulk-Ras, A., Beijersbergen, A. and Hooykaas, P. J. (1995). Trans-kingdom T-DNA transfer from Agrobacterium tumefaciens to Saccharomyces cerevisiae. *EMBO J* **14**, 3206-3214. Cardozo, T. and Pagano, M. (2004). The SCF ubiquitin ligase: insights into a molecular machine. *Nat Rev Mol Cell Biol* **5**, 739-751.

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

Gelvin, S. B. (2000). Agrobacterium and plant genes involved in T-DNAtransfer and integration. Annu Rev Plant Physiol Plant Mol Biol **51**, 223-256.

Gelvin, S. B. (2003). Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiol Mol Biol Rev* **67**, 16-37.

Gouka, R. J., Gerk, C., Hooykaas, P. J., Bundock, P., Musters, W., Verrips, C. T. and de Groot, M. J. (1999). Transformation of Aspergillus awamori by Agrobacterium tumefaciens-mediated homologous recombination. *Nat Biotechnol* **17**, 598-601.

de Groot, M. J., Bundock, P., Hooykaas, P. J. and Beijersbergen, A. G. (1998). Agrobacterium tumefaciens-mediated transformation of filamentous fungi. *Nat Biotechnol* **16**, 839-842.

Hooykaas, P. J., Hofker, M., den Dulk-Ras, H. and Schilperoort, R. A. (1984). 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* **11**, 195-205.

Hooykaas, P. J. J. and Beijersbergen, A. G. M. (1994). The virulence system of Agrobacterium tumefaciens. *Annu Rev Phytopathol* 32, 157-179.

Hua, Z. and Vierstra, R. D. (2011). The cullin-RING ubiquitin-protein ligases. *Annu Rev Plant Biol* **62**, 299-334.

Jarchow, E., Grimsley, N. H. and Hohn, B. (1991). virF, the host-range-determining virulence gene of Agrobacterium tumefaciens, affects T-DNA transfer to Zea mays. *Proc Natl Acad Sci U S A* **88**, 10426-10430.

Jonkers, W. and Rep, M. (2009). Lessons from fungal F-box proteins. *Eukaryot Cell* 8, 677-95.

Jurado Jacome. (2011). Ph.D thesis, Leiden University. Agrobacterium infection: translocation of virulence proteins and role of VirF in Host Cells.

Kumar, S. V., Misquitta, R. W., Reddy, V. S., Rao, B. J. and Rajam, M. V. (2004). Genetic transformation of the green alga-Chlamydomonas reinhardtii by Agrobacterium tumefaciens. *Plant Sci* **166**, 731-738.

Melchers, L. S., Maroney, M. J., den Dulk-Ras, A., Thompson, D. V., van Vuuren, H. A., Schilperoort, R. A. and Hooykaas, P. J. (1990). Octopine and nopaline strains of Agrobacterium tumefaciens differ in virulence; molecular characterization of the virF locus. *Plant Mol Biol* **14**, 249-259.

Michielse, C. B., Hooykaas, P. J., van den Hondel, C. A. and Ram, A. F. (2005). Agrobacteriummediated transformation as a tool for functional genomics in fungi. *Curr Genet* **48**, 1-17.

Nakayama, K. I. and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* **6**, 369-381.

Otten, L., Piotrowiak, G., Hooykaas, P., Dubois, M., Szegedi, E. and Schell, J. (1985). Identification of an Agrobacterium tumefaciens pTiB6S3 vir region fragment that enhances the virulence of pTiC58. *Mol Gen Genet* **199**, 189-193.

Regensburg-Tuink, A. J. and Hooykaas, P. J. (1993). Transgenic N. glauca plants expressing bacterial virulence gene virF are converted into hosts for nopaline strains of A. tumefaciens. *Nature* **363**, 69-71.

Schrammeijer, B., Hemelaar, J. and Hooykaas, P. J. (1998). The presence and characterization of a virF gene on Agrobacterium vitis Ti plasmids. *Mol Plant Microbe Interact* **11**, 429-433.

Schrammeijer, B., Risseeuw, E., Pansegrau, W., Regensburg-Tuink, T. J., Crosby, W. L. and Hooykaas, P. J. (2001). Interaction of the virulence protein VirF of Agrobacterium tumefaciens with plant homologs of the yeast Skp1 protein. *Curr Biol* **11**, 258-262.

Schrammeijer, B. (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.

- Tzfira, T. and Citovsky, V. (2000). From host recognition to T-DNA integration: the function of bacterial and plant genes in the Agrobacterium-plant cell interaction. *Mol Plant Pathol* **1**, 201-212.
- Tzfira, T., Vaidya, M. and 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. and Citovsky, V. (2004). Involvement of targeted proteolysis in plant genetic transformation by Agrobacterium. *Nature* **431**, 87-92.
- Vergunst, A. C., Schrammeijer, B., den Dulk-Ras, A., de Vlaam, C. M., Regensburg-Tuink, T. J. and Hooykaas, P. J. (2000). VirB/D4-dependent protein translocation from Agrobacterium into plant cells. *Science* **290**, 979-982.
- Vierstra, R. D. (2009). The ubiquitin-26S proteasome system at the nexus of plant biology. *Nat Rev Mol Cell Biol* **10**, 385-97.
- Ward, D. V., Zupan, J. R. and Zambryski, P. C. (2002). Agrobacterium VirE2 gets the VIP1 treatment in plant nuclear import. *Trends Plant Sci* **7**, 1-3.
- Welchman, R. L., Gordon, C. and Mayer, R. J. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nat Rev Mol Cell Biol* **6**, 599-609.
- Zaltsman, A., Krichevsky, A., Loyter, A. and Citovsky, V. (2010). Agrobacterium induces expression of a host F-box protein required for tumorigenicity. *Cell Host Microbe* **7**, 197-209.
- Zhu, J., Oger, P. M., Schrammeijer, B., Hooykaas, P. J., Farrand, S. K. and Winans, S. C. (2000). The bases of crown gall tumorigenesis. *J Bacteriol* **182**, 3885-3895.
- Zonneveld, B. J. M. (1986). Cheap and simple yeast media. *J Microbiol Methods* 4, 287-291.
- Zupan, J., Muth, T. R., Draper, O. and Zambryski, P. (2000). The transfer of DNA from Agrobacterium tumefaciens into plants: a feast of fundamental insights. *Plant J* **23**, 11-28.