Functional analysis of agrobacterium virulence genes

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Chapter 2

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

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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 ubiquitin-mediated 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/MATα his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0*) 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.

Table 1. Deletion strains lacking F-box proteins used in this study.

	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	*YLR352W*	*YLR352W*

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 µg/µl). 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. Aliquots 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 µl 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.

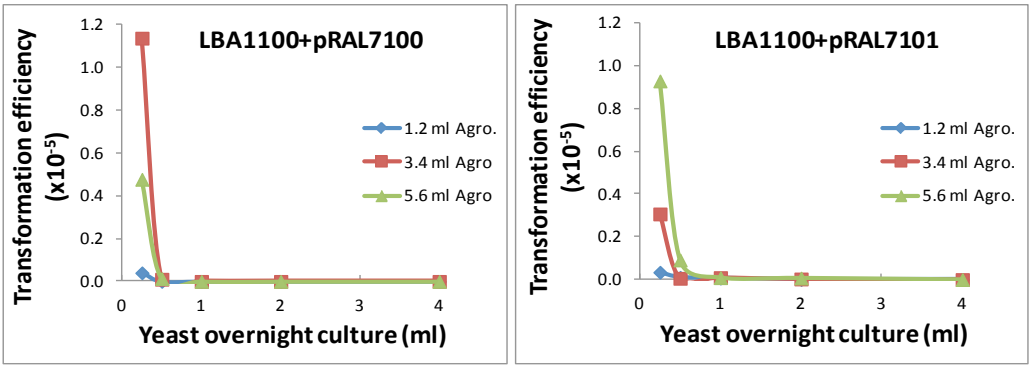

Figure 1. The effect of varying the amount of *Agrobacterium* and yeast cells on AMT efficiency. The indicated amounts of an overnight culture of *Agrobacterium* LBA1100 containing either plasmid pRAL7100 or plasmid pRAL7101 and of yeast strain BY4743 were used to prepare subcultures for co-cultivation.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

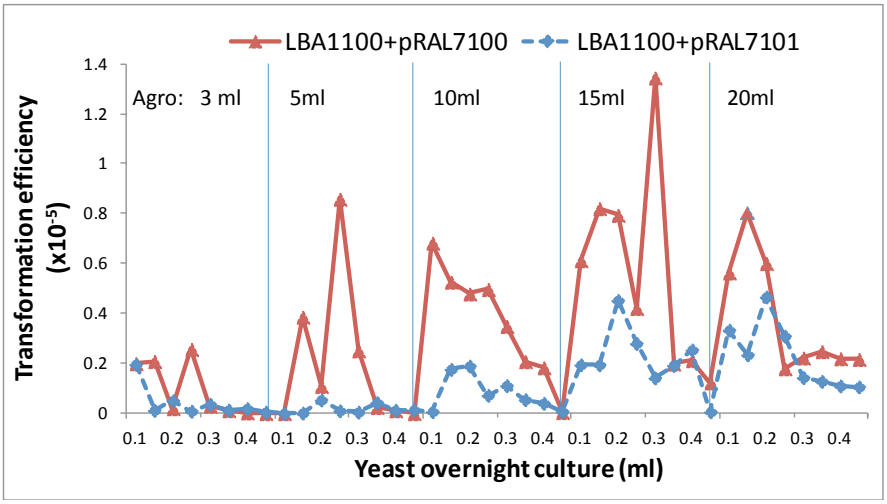

Figure 2. The effect of increased amounts of *Agrobacterium* and decreased amounts of yeast cells on AMT efficiency. The indicated amounts of an overnight culture of *Agrobacterium* LBA1100 containing either pRAL7100 or pRAL7101 and of yeast strain BY4743 were used to prepare subcultures for co-cultivation. 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						
BY4743	100	56	10	100	89	38
ELA1	93	120	47	683	458	48
YDR306C	64	88	14	130	204	17
Experiment 3						
BY4743	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						
BY4743	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.

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