Agrobacterium infection : translocation of virulence proteins and role of VirF in host cells
Jurado Jácome, E.

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CHAPTER 7

Summary and General Discussion

Agrobacterium tumefaciens, the causative agent of crown gall disease, is a natural plant genetic engineer that adopted a type IV secretion system (T4SS) for interkingdom DNA and protein transport. The T-DNA becomes integrated in the host genome and the T-DNA encoded genes are subsequently expressed, resulting in a disturbed hormone balance and the formation of crown gall tumors. The VirB/D4 T4SS, encoded by 11 *virB* genes and *virD4,* is a multiprotein complex that spans the bacterial envelope, and data suggest that both T-DNA and effector virulence proteins are transferred through this channel into the host cell (Vergunst *et al*., 2000; Schrammeijer *et al.,* 2003; Vergunst *et al.,* 2003; Vergunst *et al.,* 2005; Fronzes *et al.,* 2009). Two of these translocated virulence proteins are the single-stranded DNA binding protein VirE2 and the F-box protein VirF (Vergunst *et al*., 2000). In agreement with a role inside the host during infection for VirE2 and VirF, several interacting plant proteins have been identified (Schrammeijer *et al.,* 2001; Tzfira *et al*., 2002; Dafny-Yelin*,* Levy and Tzfira, 2008). It was assumed that also the relaxase protein VirD2, which is firmly attached to the 5'-end of the T-DNA, and for which plant interactors had been identified (Ballas and Cytovsky, 1997; Deng *et al.,* 1998), and VirE3, which has a similar C-terminal RxR transport motif as suggested for VirF and VirE2 (Vergunst *et al.,* 2000), were translocated effectors, but direct evidence was lacking. In **Chapter 2** we describe the strategy followed to prove direct protein translocation of VirD2 and VirE3 into host cells. Also we studied the possible transfer of the VirE2 chaperone, VirE1. For this we used the Cre recombinase system as a reporter to detect protein translocation into host cells (CRAfT), in which Cre recombinase is expressed in bacteria as a fusion to bacterial proteins to detect their translocation into host cells. Here, we constructed N-terminal translational fusions of the *A. tumefaciens* virulence proteins VirD2, VirE1, VirE3, and the last 50 C-terminal amino acids

of VirE3 to Cre, and expressed these in *A. tumefaciens*. To detect protein transport, we used two different *Arabidopsis* reporter transgenic lines, one in which Cre- mediated excision results in expression of a neomycin phosphotransferase (*nptII*) gene, leading to kanamycin resistance (Vergunst *et al*., 2000), and one in which Cre activity leads to GFP expression (Vergunst *et al*., 2005). Also, the yeast strain LBY2 was used in which Cre excision of a *lox*-flanked *URA3* gene resulted in 5-FOA resistance (Schrammeijer *et al.,* 2003).

We analyzed the transfer of N-terminal fusions of VirD2, VirE1, VirE3 and the last 50 C-terminal amino acids of VirE3 to Cre from *A. tumefaciens* into both yeast and plant cells. Although we were unable to reproducibly detect protein transfer of a full-length Cre::VirE3 fusion into plant cells, VirD4-dependent translocation into yeast cells was shown (Schrammeijer *et al.,* 2003). Interestingly, transport of the C-terminal 50 amino-acids of VirE3 was detected equally well in plants (Vergunst *et al.,* 2005) as in yeast, suggesting that interaction of VirE3 with host proteins (García-Rodríguez, Schrammeijer and Hooykaas, 2006; Lacroix *et al.,* 2005) may sequester the protein and prevent its entry into the nucleus, and thereby its detection in the CRAfT assay. A difference in yeast and plant proteins interacting with VirE3 may explain the observed difference in detection of transport. Our data supported the presence of a C-terminal translocation signal in VirE3, as previously found for VirF and VirE2 (Vergunst *et al*., 2000; Schrammeijer *et al*., 2003) and give evidence that VirE3 is a genuine T4SS effector.

VirE1 is a chaperone of VirE2, and prevents premature VirE2 assembly in the bacterial cell (Deng *et al*., 1999; Sundberg *et al*., 1999; Zhao *et al*, 2001). Since VirE3 is transcribed from the same operon as VirE2 (García-Rodríguez, Schrammeijer and Hooykaas, 2006), we analyzed whether VirE1 was needed for VirE3 translocation. Transfer of Cre::VirE3-50C from a *virE1* mutant was detected with equal efficiency as translocation from a wild type strain, supporting previous findings, which indicated that VirE1 is not essential for recruitment of VirE2 by the T4SS (Vergunst *et al.,* 2003, Atmakuri *et al.,* 2003). In addition, we found no indication for transfer of VirE1 into yeast or plant cells, which is in line with the absence of the C-terminal consensus transport signal established for effectors of the VirB/VirD4 T4SS (Vergunst *et al.,* 2005).

Translocation of VirD2 could not be reproducibly detected using an *Arabidopsis* reporter line with kanamycin resistance as a read-out. However, a highly efficient *Arabidopsis* reporter line, using GFP as a read out, allowed the reproducible detection of VirD4-dependent protein translocation of full length VirD2 in the absence of T-DNA, although at low efficiency (Vergunst *et al*., 2005). In **Chapter 2** we show in addition that a Cre::VirD2 fusion protein is transported into plant cells both in the presence and absence of T-DNA with equal efficiency, indicating that the presence of a VirD2 DNA substrate does neither decrease nor increase the detection of protein translocation. This could be due, however, to the fact that

the Cre-VirD2 fusion protein is not able to efficiently bind and nick the T-DNA as does VirD2 alone. Therefore, we analyzed whether a Cre-VirD2 fusion was able to complement a *virD2* mutant for DNA transfer. We found that a Cre-VirD2 fusion protein was able to complement a *virD2* mutant for T-DNA transfer, but only at 2% of the efficiency of full length VirD2, which indicates that the function of the VirD2 protein is greatly reduced by the presence of Cre sequences. Together, the data suggest that VirD2 is a T4SS substrate, and carries a transport signal that is recognized by the T4SS.

In summary, VirE3 and VirD2 are translocated effector proteins that require a functional T4SS for their translocation (Vergunst *et al.,* 2005; Schrammeijer *et al.,* 2003). The finding that VirD2 is transported into host cells in the absence of a DNA substrate, suggests that conjugation systems are actually protein transport systems, in which a coupling protein assists the translocation into recipient cells of DNA along with effector proteins (Regensburg-Tuïnk and Hooykaas, 1993; Vergunst *et al.,* 2000; Cascales and Christie, 2004b, Luo *et al.,* 2004).

The translocated *A. tumefaciens* effector protein VirF embodies the major subject of this thesis. It was shown previously that VirF is a host range determinant and necessary for full virulence on several plant species (Hooykaas *et al*., 1984; Melchers *et al*., 1990). It carries an N-terminal F-box domain, and *in vitro* experiments showed the interaction of VirF via this domain with the *Arabidopsis* S-phase kinase associated protein (ASK1), a subunit of the Skp1-Cullin-Fbox (SCF) E3 ubiquitin (Ub) ligase (Schrammeijer *et al.,* 2001). The SCF complex is an important protein complex involved in targeting of substrates designated for protein degradation in eukaryotic organisms (Hershko and Ciechanover, 1998). In **Chapter 6** we searched for evidence that VirF interacts with members of the SCF complex *in planta* by co-precipitation studies. To this end, we constructed *Arabidopsis thaliana* cell cultures expressing hemagglutinine (HA)-tagged proteins of wild type VirF or $\text{VirF}_{(\text{LP-AA})}$, an F-box mutant that has lost its ability to interact with ASK1 and was attenuated in virulence assays (Schrammeijer *et al.,* 2001; Chapter 2). Immunoprecipitation of HA-VirF from cell cultures resulted in co-precipitation of the SCF subunits ASK1 and Cullin1 (CUL1), validating the interaction of VirF with the ASK1 protein *in vivo,* and showing in addition that CUL1 is part of the complex. In a fast control process, most probably protein ubiquitination leads to high-speed protein destruction. Switching of F-box proteins in a SCF core might regulate which proteins, to which level and where they should be degraded, therefore availability of all involved subunits through all the sub-cellular space might be required. Although VirF itself does not appear to contain any typical nuclear localization signal (NLS), previous studies by Tzfira *et al.,* (2004) showed that VirF localized to the plant cell nucleus of transiently transformed cells, together with one of its proposed targets for degradation, the VirE2 interacting protein VIP1. Our immunolocalization analysis revealed that both HA-CUL1 and

HA-VirF were localized not only in the nucleus but also in the cytoplasmic fraction of epitope-tagged cell lines. These results are in line with previous findings that show that the core proteins of the E3 ubiquitin ligases, ASK1, CUL1 and HRT1, are present both in the plant cell nucleus and in cytoplasmic space (Blondel *et al.*, 2000). Similarly, depending on the location of their substrates, different F-box proteins (FBP) may display distinct patterns of sub-cellular localization to pursue their specific recognition. For example, a study by Tao *et al.,* (2005) revealed that nuclear Aux/IAAs are recruited into proteolytically active nuclear protein bodies containing SCF^{TIR1}, COP9 signalosome (CSN) and 26S proteasome components, indicating the nuclear co-localization of distinct subunits of the protein degradation system and their substrates. The capacity of VirF to interact *in vivo* with the two main core proteins of a plant assembled SCF complex, ASK1 and CUL1, strongly supports our hypothesis that VirF plays a role *in planta* as FBP, most likely mediating the ubiquitination of proteins targeted for degradation via the proteasome as part of an SCF complex either in the nucleus or in the plant cell cytoplasm. It is possible that during its journey to the plant cell nucleus, VirF may interact with ASK1 and CUL1 also in the cytoplasm and recognize targets as protein precursors. However, further study is required to determine the co-localization of HA-VirF and its target proteins, and the direct role of VirF in the degradation of these yet unknown targets inside host cells.

Identification of putative target proteins of VirF should teach us more about the role of VirF in the infection process of *Agrobacterium.* Therefore, in **Chapter 3,** we aimed to identify the eukaryotic binding partners of VirF. Using VirF as bait, we screened an *A. thaliana* cDNA library for interactors of VirF. Earlier, a similar screening with full length VirF had exclusively identified ASK1 and ASK2 as interacting proteins (Schrammeijer *et al.*, 2001). Since these proteins interact with VirF through the F-box domain, we performed a yeast two-hybrid screening with a version of VirF lacking the F-box domain (VirFΔ42N), attempting to increase the chance of identifying other interactors. In addition, we also performed a screen with full length VirF as bait. Our further analysis showed that, in contrast to results by Schrammeijer *et al.* (2001), a high diversity of different interacting clones was obtained using both VirF and VirFΔ42N. In addition, we did not identify ASK proteins as exclusive interactors with full length VirF. The difference in yeast systems (high plasmid copy number vs. low copy number) and strains, may have accounted for the observed differences between the two studies. It is possible that due to conditions of lower stringency that are required for the yeast strain (PJ694-A, James *et al.,* 1996) and the high copy number of the plasmids pAS2.1 and pACT2, our screen resulted in a higher background. However, these conditions could also have led to a more efficient two-hybrid system in which also weakly interacting clones were detected. In our screen we identified 21 clones representing 18 different genes encoding for proteins that interacted with VirFΔ42N. One protein, epithiospecifier protein-ESP, was

identified 3 times, and a Lon protease like protein was identified twice. The VIP1 protein, which was found to interact with VirF (Tzfira *et al.,* 2004), was not present among those 21 proteins. To confirm our yeast data, we performed GST pull down assays by which we confirmed that five of these proteins bound directly to VirF *in vitro*. These five protein interactors of VirF (PIFs) represent 3-deoxy-7-phosphoheptulonate 7-phosphate (DAHP) synthase 2 (DHS2, PIF1), a Lon protease like protein (PIF2), a putative pirin like protein (PIF3), the vacuolar H+ ATPase subunit B3 (VHA-B3, PIF4) and the branched-chain amino-transferase (BCAT4, PIF5). Some of these interactors have predicted localization in the chloroplast or mitochondrion. Such localization pattern may not match with the localization of VirF. However, other studies have shown the interaction of effectors with organelle proteins (Caplan *et al*., 2008). It is envisaged that such interaction may occur in the cytosol immediately after protein synthesis, followed by proteolysis in the cytosol by the proteasome machinery.

Interaction was seen with a subunit of the vacular H^+ ATPase-VHA-B3 that is involved in control of the intracellular pH. Its degradation may affect as well basic cellular processes. Three clones represent the ESP protein, a defense protein involved in the response to pathogen attack. This interaction unfortunately could not be confirmed in our experiments due to difficulties with its heterologous expression. The BCAT4 gene is involved in glucosinolates biosynthesis. Breakdown of the ESP and BCAT4 proteins may reduce the level of the plant defense response. Also, the pirin like protein was shown to have a role in the modulation of programmed cell death and stress responses (Orzaez *et al*., 2001).

Interestingly, recently a survey in *Arabidopsis* revealed a series of proteins associated to metabolism, stress responses, signal transduction and transcription that are effectively targeted by ubiquitination *in planta* (Manzano *et al*., 2008). Among these, an ATPase subunit B1 (At1g76030) was found. Alignment of this protein with our identified target VHA-B3 (At1g20260) by Clustal analysis (Larkin *et al.,* 2007), depicted high homology, which suggests that both ATPase subunits may have similar sequences that are ubiquitinated as signal for protein degradation. Similarly, a Lon protease homolog 1 (At5g47040) was also identified as an ubiquitinated protein in *Arabidopsis* (Manzano *et al*., 2008), but in contrast to the findings for VHA-B3, this protein shares very low homology with the Lon protease-like protein we identified (At1g75460). As VirF is a host range determinant required for full virulence on certain host plants only (Hooykaas *et al*., 1984; Melchers *et al*., 1990), our results strongly suggest that some or all of the proteins we identified may be targeted for proteolysis via VirF recognition and enhance *Agrobacterium* virulence in specific hosts, either by stimulating the shifting of metabolic processes or by affecting the plant defense and cell death control towards bacterial benefit and tumor proliferation in the host.

We were unable to find a common motif in the identified PIFs by sequence comparison that might represent an interaction domain with VirF. Further detailed mutational analysis of each PIF is therefore required to determine more precisely the interaction domains with VirF, and the sites for ubiquitination, for example by mass spectrometry (Marotti *et al.,* 2002). Reversely, we tried to identify the sequence motifs of VirF that are involved in substrate recognition. In **Chapter 4** we evaluated the interaction of a set of N-terminal deletions of VirF with the PIF1, PIF2, PIF3 and PIF4 proteins. After deletion of the large region containing from aa 1 to 166 in VirFΔ166N, binding to substrates no longer occurs. All substrates were still bound by VirFΔ42N and remarkably some of the PIF proteins showed even stronger interaction with VirFΔ42N compared to full length VirF. The data further suggested that two different regions may be involved in protein recognition but that this depended on the nature of the target protein. An important target recognition domain may partially be located between aminoacids (aa) 75 and 100, but the interaction was stronger when aa 42 to 75 were also present (VirFΔ42N). On the other hand, deletion of the VirF residues 100-125 completely abolished the VirF recognition of all the four PIFs. However, the weak binding of PIF2 and PIF3 found with VirFΔ145N but not with VirFΔ100N or VirFΔ125N, suggests that deletion of the N-terminal 145 aa releases a second target interaction domain in VirF that must be at least partially located between aa 145 and aa 166. These results suggests that the central domains in VirF may display more relevance for the mediation of protein-protein interaction than those present at either the N-terminal or C-terminal borders of the protein sequence. Further *in silico* analysis of VirF indicated that no particular FBP domains such as WD40, LRR or Kelch repeats were present.

Although the precise role of VirF is not known, it has been found that it may localize to the plant cell nucleus and interacts with the VIP1 protein, destabilizing in yeast and plant cells both VIP1 and its binding partner VirE2. Therefore, it has been hypothesized that VirF may function in the decoating of the T-strand, which may be required for integration into the host cell genome (Tzfira *et al*., 2004). In **Chapter 5** we used yeast to find evidence for SCF dependent protein degradation of the PIFs identified in **Chapter 3** by VirF *in vivo.* To develop the system we used VIP1 as a control protein, which is degraded by VirF (Tzfira *et al*., 2004). To this end, a gene encoding a histidine (His)-tagged VirF protein was cloned under the *GAL1* inducible promoter and introduced into yeast cells expressing hemagglutinine (HA)-tagged VIP1 or PIF proteins constitutively. At different time points after VirF induction protein levels were determined. VIP1 showed a light, but differential VirF-dependent degradation pattern independent of inhibition of de novo protein synthesis. Besides, comparison of the yeast transformed with G*al1::virF* with the control cells lacking this fusion showed a marked difference in VIP1 expression level, even before gene induction, suggesting that the sharp fluctuation of VIP1 levels could in fact correlate with the presence of VirF. Although not as

strong as for VIP1, a degradation pattern was also observed for the VHA-B3 subunit (PIF4), but not for DHS2 (PIF1) and the pirin like protein (PIF3), which maintained stable expression levels either in the presence or absence of VirF. This strengthens our idea that the VHA-B3 subunit is a target of VirF mediated proteolysis. At the same time, the other PIFs, although they interacted in the yeast two-hybrid assay and in pull down *in vitro* experiments (**Chapter 3**), may not be true *in vivo* interactors of VirF. However, possibly, a different localization of VirF and the PIFs after heterologous expression in yeast cells prevented interaction and hence proteolytic degradation. To verify whether these proteins can interact *in vivo* and are real targets for VirF-mediated degradation, further experimentation using, methods such as bimolecular fluorescence complementation assays and co-precipitation experiments with plant cells will be required.

It has been observed that several pathogens have developed mechanisms to evade or exploit the fundamental processes activated by ubiquitination, producing both ubiquitin ligases and deubiquitinases that control host responses (reviewed by Collins and Brown, 2010). For example, virulence proteins from a diversity of pathogens, i.e. from viral (Pazhouhandeh *et al*., 2006) or prokaryotic origin (*Shigella flexneri* [OspG], Kim *et al*., 2005; *Pseudomonas syringae* [AvrPtoB], Abramovitch *et al.,* 2006, Janjusevic *et al*., 2006; *Ralstonia solanacearum* [GALA], Angot *et al*., 2006; and others (reviewed by Angot *et al.,* 2007) interfere in different ways with the ubiquitination system of the eukaryotic host cell. On the other hand, some bacteria have evolved proper mechanisms to avoid or interfere with the host defense systems, as is the case for the effectors AvrPto and AvrPtoB from *Pseudomonas syringae* pv. *tomato (Pst)*, which intercept the immune response triggered by microbe associated molecular patterns (MAMPs) (Shan *et al.,* 2008). In tomato resistant plants, immunity to *Pst* is mediated by the Pto kinase, and in some cases, by associated proteins, as Fen. It has been shown that by a E3 Ub ligase C-terminal domain, AvrPtoB is able to ubiquitinate Fen, and promotes its degradation in a proteasome dependent manner, leading to disease susceptibility in Fen-expressing tomato lines (Rosebrock *et al.,* 2007). All this indicates that pathogens have evolved in line with their hosts to interfere with the specific eukaryotic systems required for their own settlement by expressing genes that mimic or regulate host protein factors involved in a variety of signalling cascades (Shames, Auweter and Finlay, 2009). In this study, we searched for evidence that *Agrobacterium tumefaciens* uses such strategies during the infection process. Our work has revealed a set of basic cell processes in which the VirF interactors here identified are involved, such as aminoacid/nucleotide biosynthesis, carbon fixation, defense-stress responses and programmed cell death. This suggests that *Agrobacterium* may have developed the capacity to shift the host metabolism by mimicking the function of an eukaryotic F-box protein (FBP) able to interact with an E3 Ub-ligase and recognize plant targets involved in relevant cell

processes, interfering in natural signalling cascades and use them towards its benefit and proliferation. Recent studies have revealed how certain virulence factors from *Salmonella* and *Shigella* species are able to function as autoregulated E3 Ub-ligases inside the host cell carrying proper substrate-recognition and catalytic domains (Quezada *et al.,* 2009; Singer *et al*., 2008), therefore, it is not unexpected that VirF could function as and FBP inside eukaryotic cells.

A. tumefaciens mediated transformation has been widely adopted, counting nowadays as one of the most used technologies in the genetic improvement of relevant commercial crops. Nevertheless, we are still far from a full understanding of this *A. tumefaciens* infection process. The research described in this thesis has contributed to our understanding of how *Agrobacterium* has evolved an effective system for translocation of effector virulence proteins that play a role inside host cells to effectively colonize its eukaryotic partners.

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