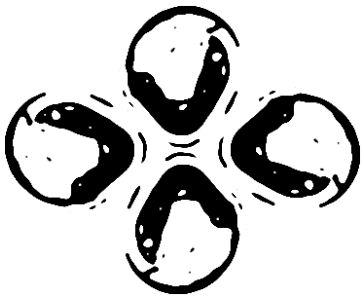
Chapter 5

Summary



Summary

The soil bacterium *Agrobacterium tumefaciens* is able to genetically transform plant cells by transferring a piece of DNA, the T-DNA, into the host cell. After transfer of the T-strand through a Type IV Secretion System (T4SS) and entry into the nucleus, the T-DNA integrates into the host chromosomal DNA. The T-strand carries genes which code for enzymes involved in the synthesis of phytohormones (auxin and cytokinin) and opines. Opines produced by the tumorous growing transformed cells can be used by *A. tumefaciens* as a source of carbon and nitrogen. This method of infection is known as *Agrobacterium*-Mediated Transformation (AMT). During the transformation process, at least five different virulence proteins, i.e. VirD2, VirD5, VirE2, VirE3 and VirF, are transferred from the bacterium into the host cell.

The virulence proteins of *A. tumefaciens* play key roles in the transformation of host cells [1]. As yet, the exact functions of these effectors in the host are not fully understood. Under laboratory conditions *A. tumefaciens* is also able to transform non-plant organisms like yeasts and fungi. This enables us to use the experimental advantages of the model yeast *Saccharomyces cerevisiae* to investigate *A. tumefaciens* virulence proteins. In this study we visualized the delivery of translocated proteins from *A. tumefaciens* to yeast and plant cells *in vivo* to gain more insight in the functions of these proteins during AMT. As genetic fusions to GFP inhibit the translocation of virulence proteins through the T4SS, we developed two alternative approaches: one method based on the BiFC (Bimolecular Fluorescence Complementation) assay [2] (Chapter 3) and one method based on the split GFP system [3] (Chapters 3 and 4; for overview see Chapter 3, Figure 1). Real time microscopy was performed to study the timeframe in which virulence induction, protein translocation and (transient) T-DNA expression occur during AMT. Additionally, *in vivo* studies on the VirE2 and the VirE3 protein (Chapters 2 and 4) were performed. In Figure 1, a schematic representation summarizes some of the main findings of this thesis.

<u>The BiFC strategy is an efficient method to detect protein translocation to yeast *in vivo* and in real time</u>

Using the BiFC strategy we studied translocation of VirE2 and VirE3 from *A. tumefaciens* to yeast (Chapter 3). As the BiFC assay is used to detect protein – protein interactions, we needed to express interaction partners of VirE2 and VirE3 in yeast host cells. As VirE2 self associates in the absence of

VirE1 [4], we chose to express VirE2 in yeast cells to visualize translocation of VirE2 from the bacterium to yeast. As an interaction partner of VirE3 we chose pBrp, a TFIIB related plant protein previously reported to bind VirE3 in yeast two-hybrid experiments [5]. VirE2 and pBrp could be stably expressed in yeast (Chapter 2 and 3) and we showed that the interactions VirE2 – VirE2 (Chapter 2, Figure 4) and VirE3 – pBrp (Chapter 3, Figure 2) could be visualized in yeast by the BiFC assay. In subsequent cocultivations we visualized delivery of VirE2 and VirE3 proteins in recipient yeast cells (Chapter 3, Figure 3). Fluorescent signals were observed after approximately one day for both VirE2 and VirE3 indicating that the BiFC strategy is an efficient method to visualize translocation *in vivo*. In these cocultivation experiments proteins were tagged with the VN173 and VC155 BiFC fragments of Venus [6], a YFP analogue. *In vitro* studies by Miyawaki *et al.* [7] have shown that Venus has, compared to other fluorophores, significantly improved maturation times of a few seconds. Hence the developed BiFC approach also enables real time visualization of protein translocation. As expected cocultivation experiments with T4SS defective *A. tumefaciens* mutants did not result in reconstituted BiFC signals in yeast host cells, as translocation of VirE2 and VirE3 is known to be T4SS-dependent (Chapter 3).

Visualization of protein translocation from *A. tumefaciens* to yeast using the split GFP system

The split GFP system can be a very powerful system to study protein translocation and therefore we applied this system to visualize protein translocation from *A. tumefaciens* to yeast. To this end, we tagged the effector protein with the eleventh helix of GFP (GFP 11) and expressed GFP lacking the eleventh helix (GFP 1-10) in yeast. After translocation of the tagged effector protein the GFP fragments self-associate resulting in a fluorescent protein. Using this strategy we successfully visualized translocation of the virulence proteins VirD2, VirD5, VirE2 and VirF to yeast (Chapter 3, Figure 6). Time-lapse microscopy showed that the GFP 11 tagged virulence proteins were translocated after approximately 1 day of cocultivation (Chapter 3, Figure 9). This time-frame of virulence protein translocation was similar to that observed using the BiFC approach. Transferred effector proteins were mostly localized at spot-shaped or filamentous structures inside the yeast cell. When using the BiFC approach, the localization of reconstituted fluorescent protein is dependent on the properties of both binding partners and thus may not reflect the true localization of the translocated protein. In contrast, experiments adopting the split GFP system do not have this drawback: the GFP 1-10 molecule is expressed all over the yeast cell and the localization of

detected fluorescence solely depends on the translocated protein.

We were interested to know whether N-terminal tagging with GFP 11 affected the activity of virulence proteins during AMT. As VirD2 is an essential protein for AMT of yeast, we compared transformation efficiencies using an *A. tumefaciens* strain expressing wild-type VirD2 and an *A. tumefaciens* strain expressing GFP 11-VirD2. Cocultivation with *A. tumefaciens* expressing GFP 11-VirD2 resulted in transformed yeast cells, although we observed a 60% drop in AMT efficiency (Chapter 3, Figure 8). This indicated that the VirD2 fusion proteins were functionally active in AMT. We subsequently investigated whether C-terminal tagging of the GFP 11-VirD2 protein with the translocation signal of VirF could have a beneficial influence on AMT, but we did not observe a positive effect on the transformation efficiency (Chapter 3, Figure 8).

Influence of T-DNA on the subcellular localization of translocated virulence proteins

Using the split GFP strategy we visualized the subcellular localization of translocated virulence proteins VirD2, VirD5, VirE2 and VirF in the presence and absence of T-DNA (Chapter 3). We showed that the absence of T-DNA did not affect the localizations of translocated VirD5, VirE2 and VirF. In contrast we did observe an effect on the localization of translocated GFP 11-VirD2. In the presence of T-DNA, VirD2 localized in a compact dot-shaped structure inside the cell, whereas in the absence of T-DNA, VirD2 localized all over the nucleus (Chapter 3, Figure 6). Complementation of a T-DNA deficient *A. tumefaciens* strain with a binary plasmid harboring T-DNA restored the localization of translocated GFP 11-VirD2 in discrete dots (Chapter 3, Figure 7).

The split GFP system can be used to visualize delivery of virulence proteins into plant cells

As mentioned above, the split GFP strategy proved to be successful to visualize translocation of virulence proteins into yeast cells. Since plants are the natural hosts of *A. tumefaciens*, we investigated whether this strategy could also be utilized to visualize delivery of virulence proteins to plant cells. For this purpose transgenic tobacco SR1 lines were generated constitutively expressing GFP 1-10. These plants were agroinfiltrated with *A. tumefaciens* expressing GFP 11-tagged virulence proteins and, 24 hours after infiltration, fluorescence could be detected in the leaf epidermal cells (Chapter 4, Figure 6). Fluorescence was detected in the cytoplasm after agroinfiltrations with bacterial strains expressing

GFP 11-tagged VirD5. Agroinfiltrations with bacteria expressing GFP 11-VirD2 resulted in nuclear fluorescence. Fluorescence was observed in both the nucleus and the cytoplasm after agroinfiltrations with strains expressing GFP 11-VirE2 and GFP 11-VirF.

To determine whether expression of genes on the T-DNA occurs in the same time frame as protein translocation, we injected tobacco leaves with an *A. tumefaciens* strain harbouring YFP-AGC3-4 under control of the 35S promoter and terminator on its T-DNA. In this way we could detect strong YFP fluorescence around 24 hours after agroinfiltration (Chapter 4, Figure 7).

The VirE2 protein co-localizes and physically interacts with microtubules in yeast

During AMT the virulence protein VirE2 is translocated through the T4SS from *A. tumefaciens* into the host cell. To study the fate of VirE2 in *S. cerevisiae* cells we expressed YFP-VirE2, CFP-VirE2 and VirE2-GFP fusion proteins in yeast. Microscopic studies showed that all VirE2 fusion proteins (N-terminal fusions as well as the C-terminal GFP fusion to VirE2) can be stably expressed in yeast and they aggregate as thread-like structures within the yeast cells (Figure 1, Chapter 2). These filamentous structures strongly resemble the yeast microtubule structures. To examine whether VirE2 co-localizes with microtubules, we expressed CFP-VirE2 in yeast marker strain MAS101 [8] which expresses GFP-Tub1p (Chapter 2, Table 1). The Tub1 protein is a sub-unit of the microtubule structure in yeast [9]. Confocal microscopy showed that CFP-VirE2 indeed co-localizes with GFP-Tub1 (Chapter 2, Figure 2A-C). Treatment of these yeast cells with the microtubule-destabilizing agent benomyl [10][11] led to the disruption of microtubule and VirE2 filaments which still co-localized together (Chapter 2, Figure 3). Similar observations were made in *Arabidopsis* protoplasts, where expression of YFP-VirE2 also resulted in filamentous structures (Chapter 4, Figure 1A and B). Treatment of these protoplasts with the microtubule-destabilizing agent oryzalin [12] resulted in a severe change in localization of YFP-VirE2: the thread-like structures were either completely abolished or considerably shortened (Chapter 4, Figure 1C and D).

It has been reported that VirE2 proteins bind to microtubules in a cell-free *Xenopus* oocyte extract [13]. To investigate whether *in vivo* VirE2 physically interacts with the Tub1p sub-units of microtubules in yeast we adopted a sensitized emission approach to measure FRET (reviewed in [14]) between mTurquoise-Tub1p and YFP-VirE2. In this way we validated the interaction between VirE2 and Tub1p in yeast (Chapter 2, Figures 6).

VirE2 movement along microtubules: ectopic expression *versus* translocation

Salman *et al.* have shown that "animalized" VirE2 proteins are able to move along microtubules *in vitro* [13]. To study movement of VirE2 in living yeast cells, we used two different methods (Chapter 2). First, making use of the inducible *GAL1* promoter we induced CFP-VirE2 expression and followed expressed proteins over time. In a second setup we induced fluorescence of photoactivatable GFP (PA-GFP) [15][16] fused to VirE2 and followed the activated fluorescent VirE2. Neither of the two methods allowed detection of VirE2 fusion protein movement. On the other hand, we were able to visualize movement of VirE2 which was translocated from *A. tumefaciens*. Using the BiFC and split GFP approaches to visualize protein translocation of VirE2 fusion proteins, we were able to detect elongation of a dot-like fluorescent structure into a filamentous structure over time indicating directional movement of VirE2 (Chapter 3, Figure 4A and Figure 9C). This observed elongation suggests a role for VirE2 in directional movement of the T-complex along the microtubules towards the nucleus.

The inability to detect movement of host cell-expressed VirE2 proteins might be due to a higher abundance of VirE2 proteins when ectopically expressed in yeast, compared to the amount naturally translocated during AMT. This might lead to direct colonization of the entire microtubule structures and as a result movement cannot be detected. It can also be argued that the presence of other translocated proteins might have an effect on the behavior of VirE2 in the host. In any case, this difference underlines the importance of studying the localization of translocated instead of ectopically expressed virulence proteins in the host cell.

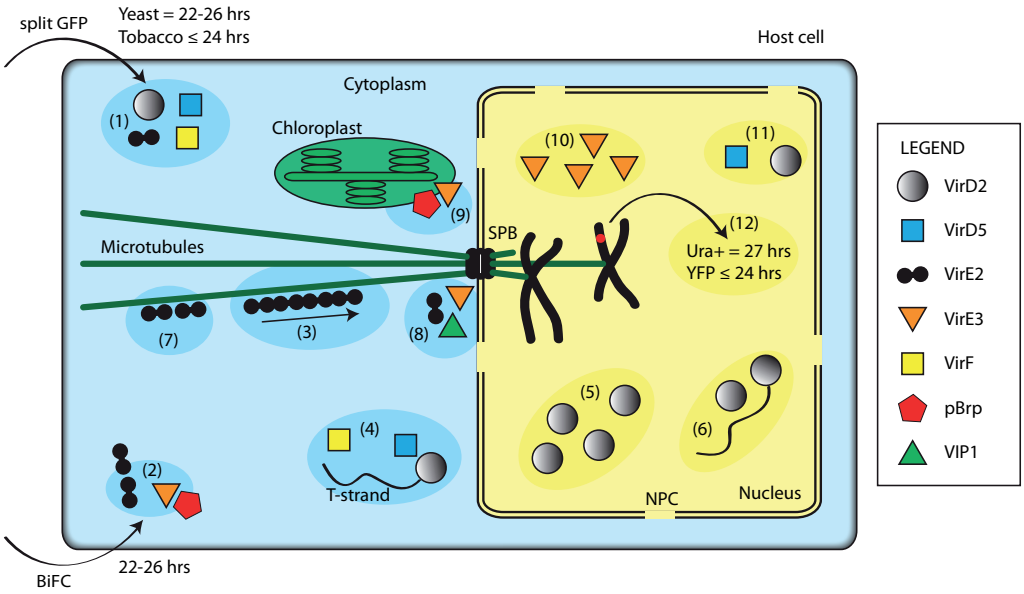


Figure 1: Schematic summary of the results of this study. (1) Using a split GFP strategy, protein translocation to yeast was detected within a time frame of 22 – 26 hours and to plants within 24 hours. (2) Using a BiFC strategy, VirE2 and VirE3 translocation to yeast was detected within a time frame of 22 – 26 hours. (3) Using both the split GFP strategy and the BiFC strategy, directional elongation of VirE2 derived fluorescent signal was detected. (4) Upon translocation, VirD2 (in the presence of T-DNA), VirD5 and VirF were found in dot-shaped structures. (5) In the absence of T-DNA translocated VirD2 localized in the nucleus of yeast cells. (6) In agroinfiltrated tobacco cells, translocated VirD2 was detected in the nucleus. (7) Expression studies in yeast have shown that VirE2 self-associates and physically interacts with the Tub1p sub-unit of microtubules. (8) Interactions of VirE2 with VIP1 and VirE3 were found at the spindle pole body in yeast. (9) Interaction of VirE3 with pBrp takes place at the chloroplasts in protoplasts. (10) VirE3 expressed in *Arabidopsis* protoplasts localizes in the nucleus. (11) VirD2 and VirD5 expressed in yeast are detected in the nucleus. (12) During AMT, expression of genes on the T-DNA was first detected after 27 hours in yeast. In tobacco cells expression of genes on the T-DNA was detected after 24 hours. NPC, nucleopore complex; SPB, spindle pole body.

Visualization of the interactions of VirE2 with VirE1, VIP1 and with itself in yeast

Expression of CFP-VIP1 and YFP-VirE3 in yeast resulted in nuclear fluorescence and fluorescence detected at the spindle pole body, respectively (Chapter 2, Figure 8A and B). Expression of GFP-VirE1 in yeast was detected all over the cell (data not shown). Using the BiFC assay we showed that VirE2 interacts with VirE1 (data not shown), VIP1 (Chapter 2, Figure 8C) and with VirE3 (Chapter 2, Figure 8D). Additionally, co-expression of CFP-VirE2 and YFP-VirE1 resulted in inhibition of CFP-VirE2 filament formation (Chapter 2,

Figure 7), which is in accordance with previous results by Krispin *et al.* [4] who found that VirE2 self associated in the absence of VirE1. This reported VirE2 self association was validated in yeast using the BiFC assay (Chapter 2, Figure 4) and the acceptor photobleaching approach to measure FRET (reviewed in [14]) (Chapter 2, Figure 5). The VirE2 – VIP1 and VirE2 – VirE3 interactions both co-localized with the spindle pole body marker Spc42 (Chapter 2, Figure 8C and D). These findings suggest a possible role of yeast host factors and VirE3 located at the spindle pole bodies in nuclear uptake of VirE2.

In vivo visualization of VirE3 and its interaction with plant host factors in *A. thaliana* protoplasts

YFP-VirE3 expressed in *Arabidopsis* protoplasts has a nuclear localization (Chapter 4, Figure 2A). This subcellular localization differs from that of YFP-VirE3 in yeast, which was present at the spindle pole body (Chapter 2, Figure 9B). This difference in localization observed in the two organisms suggests that the import machinery used in plants for nuclear uptake of VirE3 is not used in yeast or that in yeast VirE3 is trapped by proteins present at the spindle pole body. Using a yeast two-hybrid (Y2H) screen, García-Rodríguez *et al.* detected an interaction of VirE3 with four plant factors [5]: the Impα-4 importin, the Kapα importin, the plant specific TFIIB related protein pBrp, and the component of the COP9 signalosome Csn5. Using a BiFC approach in protoplasts we validated the VirE3 – Impα-4 and VirE3 – pBrp interactions *in vivo*.

A nuclear signal was detected upon expression of YFP-Impα-4 in protoplasts (Chapter 4, Figure 2). Expression of YC-VirE3 and YN-Impα-4 resulted in a reconstituted YFP signal, also localized inside the nucleus (Chapter 4, Figure 3).

Expression of YFP-pBrp led to a (partially) similar localization to that of plastids in protoplasts (Chapter 4, Figure 2). Localizations of BiFC signals resulting from the VirE3 – pBrp interaction were comparable to those observed in protoplasts expressing YFP-pBrp. These results confirm the results of García-Rodríguez *et al.* [5] and suggest that, upon binding to VirE3, the pBrp – VirE3 complex may travel to the nucleus and pBrp may affect transcription of genes beneficial to AMT.

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