Functional analysis of agrobacterium virulence genes

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Chapter 1

General Introduction

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Agrobacterium tumefaciens is a gram negative rod-shaped aerobic soil bacterium, which causes crown gall disease in plants. Tumor-like structures, present at both the root crown and stems of infected plants, are characteristic for this disease. *A. tumefaciens* was first described in 1907 and is a plant pathogen with a broad-host range which can infect more than 600 dicotyledonous plant species (Cleene and De Ley, 1976). *A. tumefaciens* carries an extrachromosomal plasmid, the Ti-plasmid, which is responsible for tumor formation. Upon infection of wounded plant cells a piece of this plasmid, the T-DNA, is transferred into host cells and integrated into one of its chromosomes (Hooykaas and Beijersbergen, 1994). This genetic transformation results in uncontrolled cell division and the synthesis of opines which are preferred nitrogen and carbon sources for *A. tumefaciens*. In a similar way, the related bacterium *Agrobacterium rhizogenes*, which carries the root inducing (Ri) plasmid, induces hairy root disease in dicotyledonous plants (Hooykaas and Beijersbergen, 1994; Zhu *et al*., 2000). Under laboratory conditions numerous non-plant organisms can be successfully transformed by *A. tumefaciens* such as algae (Kumar *et al*., 2004), yeasts (Bundock *et al*., 1995) and filamentous fungi (Gouka *et al*., 1999; Groot *et al*., 1998). The ability to transform the yeast *Saccharomyces cerevisiae* enables the use of the many experimental tools available for research on this organism in research on the mechanism of *Agrobacterium*-mediated transformation (AMT) of eukaryotic cells. Due to its unique ability of transkingdom gene transfer, *A. tumefaciens* has become an indispensible tool in plant and fungal molecular biology and biotechnology. In addition, the mechanism by which *Agrobacterium* infects plants also serves as an important model for understanding how pathogens recognize hosts and deliver macromolecules into target cells, resulting in disease.

1 Ti plasmid

Agrobacterium harbors a large extrachromosomal plasmid, the Tumor Inducing (Ti) plasmid of approximately 200 kb. Each strain of *Agrobacterium* induces a characteristic set of opines in the crown gall tumors which they provoke by their T-DNA genes. These can be catabolized by the cognate bacterial strains by enzymes encoded by the Ti plasmid. Ti plasmids are classified as octopine, nopaline, succinamopine or leucinopine plasmids based on the type of opines produced in the tumors by their T-DNA encoded opine synthase genes. Figure 1 shows a map of the octopine type Ti plasmid, This Ti plasmid consists of five main regions: (1) the T- region, (2) the virulence region, (3) the region for plasmid conjugative transfer, (4) the region for opine catabolism and (5) the replicator. The T-region and virulence region are required for tumorigenesis.

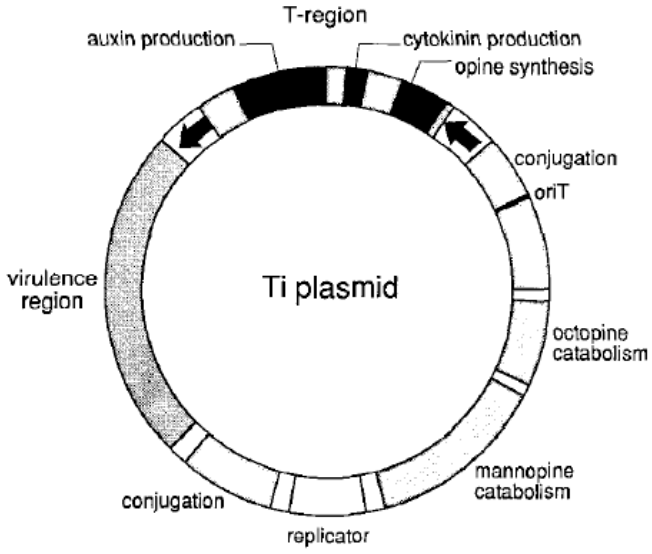

Figure 1 Schematic map of the octopine Ti plasmid (Hooykaas and Beijersbergen, 1994)

Upon infection the T-region is transferred into plant cells and integrated into their genome. The T-region is flanked by short imperfect direct repeats of 24 bp, the Left and Right border repeats. The right border of the T-region is essential for T-DNA transfer, while the left border is dispensable (Miranda *et al*., 1992). The T-region contains genes involved in auxin production (*iaaM* and *iaaH*), a gene involved in cytokinin production (*ipt*) and one or more genes involved in opine synthesis. The auxin and cytokinins produced by the transformed plant cells induce uncontrolled cell proliferation and consequently, the formation of a tumor. The opines produced serve exclusively as nutrients for *Agrobacterium* as opines cannot be utilized by most other soil bacteria; by genetic engineering, *Agrobacterium* thus creates a favorable ecological niche for itself.

Opine catabolism genes are under control of a repressor and are only induced in the presence of the opine inducer. Opines also control the conjugal transfer of the Ti plasmid. In nature the Ti plasmid becomes conjugative specifically in or near crown gall tumors due to the presence of opines there.

Genes located in the *tra* and *trb* gene clusters are responsible for the conjugal transfer of the Ti plasmid from one bacterium to another (Alt-Mörbe *et al*., 1996; Farrand *et al*., 1996). Conjugal transfer happens in the presence of a conjugative opine, but only at high cell density because it is controlled by autoinduction via the transcriptional activator TraR and which requires the acyl-homoserine lactone, *Agrobacterium* autoinducer (AAI) (Zhang *et al*., 1993; Piper *et al*., 1993).

This cell density responsive transcriptional regulation (Quorum sensing) is similar as that of the marine luminescent bacterium *Vibrio fisheri*, the paradigm quorum-sensing system (Fuqua *et al*., 1994). In *Agrobacterium*, the *traR* gene is under control of opine as it is part of an operon consisting of genes involved in opine catabolism.

The octopine virulence region is around 40 kb and is located adjacent to the left border of the T-region. It consists of a number of operons (Schrammeijer *et al*., 2000). Mutation of any of *virA*, *virB*, *virD* and *virG* operons results in avirulence, while mutation of genes in the other operons has no effect or only causes attenuated virulence on some plant species (Stachelt and Nester, 1986). All virulence genes are active in the presence of plant wound sap, which has a low pH and which contains phenolic compounds secreted by wounded plant cells such as acetosyringone (AS).

2 VirA/VirG two component regulatory system

Expression of virulence genes is initiated by activation of the VirA/VirG two component regulatory system. VirA is an inner membrane protein that senses phenolic compounds such as acetosyringone (AS) released by the plant. The sensitivity is highly increased by low pH and the presence of monosaccharides (Turk *et al*., 1991). After activation, VirA becomes phosphorylated at a histidine residue within the C-terminal half of the protein, present in the bacterial cytoplasm, then the phosphate is transferred to an aspartate residue of VirG, a cytoplasmic transcriptional regulator (Winans, 1991). Phosphorylated VirG binds to a specific 12 bp DNA sequence(vir boxes) upstream of the *vir* operons and activates the expression of the *vir* genes (Jin *et al*., 1990; Pazour and Das, 1990).

3 T-DNA production

Upon *vir* gene induction, a single stranded copy of the T-region, the T-strand, is produced and subsequently transferred to the plant cell and integrated into the plant genome. T-strand production is mediated by the virulence proteins VirD1 and VirD2 (Filichkin and Gemn, 1993). These two proteins are encoded by the *virD* operon and function together as an endonuclease that nicks at a precise site between the third and fourth bases of the bottom strands of the right and left border sequences of the T region (Jasper *et al*., 1994).The left and right border sequences are 24 bp imperfect direct repeats. Adjacent to the right border, there is a *cis*-enhancer element designated “Overdrive sequence” to which VirC1 and VirC2 specifically bind and accelerate the level of border nicking (Lu *et al*., 2009; Toro *et al*., 1989). It is thought that after nicking, the T-strand is released by a rolling-circle like replication mechanism (Waters and Guiney, 1993). The VirD2 protein remains covalently attached to the 5’-end of the nicked DNA through a tyrosine residue (Mysore *et al*., 1998). This attachment is supposed to protect

the T-strand from degradation by exonucleolytic cleavage (Dürrenberger *et al*., 1989). Besides, VirD2 contains nuclear localization signals (NLS) which help to target the T-strand into the plant cell nucleus (Howard *et al*., 1992).

4 Type IV secretion system

The T-strand is transported from *Agrobacterium* to the plant cell cytoplasm via a Type IV Secretion System (T4SS) by a mechanism that resembles that of bacterial conjugation (Christie and Vogel, 2000). Type IV secretion systems are multi subunit cell-envelope-spanning structures (Christie *et al*., 2005). The *Agrobacterium* T4SS is encoded by the *virB* and *virD* operons. The *virB* operon consists of 11 genes, *virB1* to *virB11*. The products of the *virB* genes, termed the mating pair formation (Mpf) proteins, set up a cell envelope spanning structure (Christie, 2004). The extracellular T pilus is made up mainly by the VirB2 and VirB5 proteins which also participate in attachment to the recipient cell (Lai and Kado, 1998; Schmidt-Eisenlohr *et al*., 1999; Hwang and Gelvin, 2004). Deletion of *virB1* results in severely attenuated virulence, while deletion each of *virB2* to *virB11* completely abolishes virulence (Berger and Christie, 1994). The *virD* operon consists of five genes, *virD1* to *virD5*, of which only the product of the *virD4* gene participitates in the secretion. VirD4 is an integral membrane protein and anchors to the inner membrane of *Agrobacterium* through its N-terminal region (Okamoto *et al*., 1991). VirD4 functions as a coupling protein (CP) mediating interaction between T-strand and the T4SS. This interaction is probably mediated through VirD2, which has been shown to interact with VirD4 in the yeast two hybrid assay (LIosa and O'Callaghan, 2004).

5 Translocation of virulence proteins into plant cells

Apart from the T-strand-VirD2 complex, several other virulence proteins such as VirE2, VirE3, VirD5 and VirF are translocated into plant cells via the T4SS (Vergunst *et al*., 2005). By using the Cre recombinase reporter assay for translocation (CRAFT) in which a Cre recombinase protein is fused to the N-terminus of the virulence proteins, it has been shown that the fusion proteins are translocated into plant cell independently of the T-strand (Vergunst *et al*., 2000).

The *virE* operon consists of three genes, *virE1*, *virE2* and *virE3*. The VirE2 protein is a non-sequence-specific single strand DNA-binding protein. VirE2 only binds to the T-strand after translocation into the plant cytoplasm (Ziemienowicz *et al*., 2001). There, it not only protects T-strands from nucleolytic degradation, but also it assists in targeting the T-strands to the plant cell nucleus. VirE1 is a chaperone protein of VirE2, which interacts with VirE2 in the bacterium (Deng *et al*., 1999). This interaction stabilizes VirE2 by preventing the formation of aggregates and keeps VirE2 in a proper state for translocation through the T4SS. However, VirE1 is not required for recognition of VirE2 by the T4SS (Vergunst *et al*., 2003). The VirE3 protein has a nuclear localization signal and may be a

transcription activator by interaction with plant transcription factor pBrp (García-Rodríguez *et al*., 2006).

VirD5 is another effector protein that is targeted into the plant nucleus but it is not essential for plant tumor formation (Vergunst *et al*., 2005). Recently, it was reported that VirD5 can physically interact with VirF and stabilizes VirF (Magori and Citovsky, 2011).

The *virF* gene was first identified on the octopine type Ti plasmid pTiB6 by transposon Tn1831 insertion mutagenesis (Hooykaas *et al*., 1984). *virF* mutants show strongly diminished virulence on tomato and tobacco, but not on a number of other host plants, and therefore, VirF is defined as a host-range-determining virulence protein (Jarchow *et al*., 1991; Melchers *et al*., 1990). For instance, nopaline strains, which are lacking *virF*, are avirulent on *N. glauca* (Melchers *et al*., 1990). However, these strains are virulent on transgenic *N. glauca* lines expressing the *Agrobacterium* VirF protein (Regensburg-Tuink and Hooykaas, 1993).

It was found that the translocated *Agrobacterium* virulence proteins VirE2, VirE3, VirD5 and VirF have a similar C-terminal transport signal that is recognized by the TFSS (Vergunst *et al*., 2005). The about 40 amino acid residues at the C-terminus of these proteins can mediate translocation of a Cre reporter protein from *Agrobacterium* to host cells including *Arabidopsis thaliana* and *S. cerevisiae*. Detailed mutational analysis showed a positively charged hydrophilic consensus sequence (R-X7-R-X-R-X-R) which is an important characteristic of the C-terminal transport signal for the VirB/D4 transport system (Vergunst *et al*., 2005).

6 Plant proteins involved in nuclear transport of the T-complex

Once inside the plant cell cytoplasm, VirE2 proteins bind to the T-strand along its entire length, protecting it from host nuclease attack. Nuclear import is mediated by VirD2 and VirE2, which both contain nuclear localization signals (NLS). VirD2 contains two putative nuclear localization signals, a monopartite NLS in its N-terminal part and a bipartite NLS in its C-terminal part (Rossi *et al*., 1993). The C-terminal bipartite NLS is necessary for nuclear import. Mutation of this region resulted in reduced tumorigenicity (Howard *et al*., 1992), consistent with reduced nuclear localization of the mutated VirD2 in plant cells (Ziemienowicz *et al*., 2001). Yeast two hybrid experiments showed that VirD2 can directly interact with several plant proteins which are involved in nuclear protein import. The first identified plant protein was the *Arabidopsis* importin-α (AtKAPα) (Ballas and Citovsky, 1997). Importin-α is a nuclear-transport receptor which, in most cases, functions together with importin-β. This importin alpha/beta heterodimer targets many proteins to the nuclear pore complex (NPC) and assists in their translocation across the nuclear envelope (Goldfarb *et al*., 2004). VirD2 binds to

importin-α in a C-terminal bipartite NLS dependent manner both in yeast and *in vitro*, indicating that this receptor facilitates nuclear uptake of the VirD2 protein via its NLS. In addition to Importin-α, *Arabidopsis* cyclophilins and tomato DIG3 were found to bind to VirD2 (Deng *et al*., 1998; Tao *et al*., 2004). Three isoforms of cyclophilins named Roc1, Roc4 and CypA have been shown to interact with VirD2, but the interaction domain is distinct from the NLS and endonuclease domains of VirD2 (Deng *et al*., 1998). Interruption of the interaction between VirD2 and cyclophilins by cyclosporin A inhibits *Agrobacterium*- mediated transformation of plants (Deng *et al*., 1998). This observation may suggest that cyclophilins function as chaperones to keep VirD2 in an appropriate conformation in the host cytoplasm for nuclear import. DIG3 encodes a type 2C serine/threonine phosphatase (PP2C), which specifically interacts with the C-terminal half of VirD2, and its overexpression partially inhibits nuclear import of the fused β-glucuronidase reporter protein GUS-VirD2 in tobacco BY2 protoplast cells (Tao *et al*., 2004). DIG3 negatively regulates VirD2 nuclear import probably due to dephosphorylation of VirD2. This is further supported by the observation that an *Arabidopsis abi1* mutant (lacking a PP2C activity) showed a higher susceptibility to *Agrobacterium* infection (Tao *et al*., 2004).

The VirE2 protein contains two separate bipartite NLSs; deletion of both NLSs of a GUS-VirE2 fusion protein blocked nuclear localization, whereas deletion of only one NLS partially reduced nuclear localization, suggesting that nuclear import of VirE2 is mediated by both of their NLS (Citovsky *et al*., 1992). Indeed, VirE2 directly interact with different isoforms of importin-α as shown for VirD2 (Bhattacharjee *et al*., 2008). Besides, it has been shown in a yeast two hybrid assay that two plant proteins, VIP1 (VirE2-interacting protein 1) and VIP2, interact with VirE2 (Tzfira *et al*., 2001). VIP1 is a nuclear protein containing a basic leucine zipper (bZIP) motif, which binds to AtKAPα via its NLS and therefore is imported into the cell nucleus (Tzfira *et al*., 2002). The interaction with VirE2 facilitates VirE2 nuclear import in plants as indicated by the finding that the nuclear import of VirE2 is impaired in VIP1 antisense transgenic plants (Tzfira *et al*., 2002). Later, it was found that VIP1 can associate with plant nucleosomes *in vitro* and through binding to VirE2, the T-complex may come into contact with plant nucleosomes as well (Lacroix *et al*., 2008).

The *virE3* gene is conserved in the different types of Ti and Ri plasmids (García-Rodríguez *et al*., 2006). Mutation of the gene does not lead to an obvious phenotype. The VirE3 protein has bipartite nuclear localization signals (NLS) through which VirE3 binds to *Arabidopsis* AtKAPα and is imported into the plant cell nucleus. VirE3 also interacts with VirE2, and seems to assist in the nuclear import of VirE2 (Lacroix *et al*., 2005). Thereby, VirE3 mimics the function of VIP1 and mediates the entry of the T complex into the plant cell nucleus (García-Rodríguez *et al*., 2006; Lacroix *et al*., 2005). Mutation of both NLSs of

VirE3 resulted in a mainly cytoplasmic localization of VirE2 despite that VirE3 still binds to VirE2 (Lacroix *et al*., 2005).

7 Function of VirF in the plant cell nucleus before T-DNA integration

VirF is defined as a host-range-determining virulence protein (Melchers *et al*., 1990). VirF is not essential for T-DNA transfer to many plant species, yeast and fungi (Bundock *et al*., 1995; Melchers *et al*., 1990). It does not contain NLS sequences but VirF still localizes to the plant cell nucleus (Tzfira *et al*., 2004; Jurado, 2011). VirF contains a putative F-box motif through which it interacts with the *Arabidopsis* Skp1-like (ASK) proteins ASK1, ASK2 and ASK10 (Schrammeijer *et al*., 2001). Mutation of the F-box motif by alanine substitution of two conserved leucine and proline residues abolished interaction with ASK1 (Schrammeijer *et al*., 2001). The ASK1 protein is a plant homolog of the yeast Skp1 protein and is a component of classical E3 ubiquitin ligases called SCF (Skp1-Cdc53-F box) proteins. Therefore, VirF seems to be involved in ubiquitin-mediated protein degradation.

When the T- complex arrives in the plant nucleus, several steps are necessary before T-DNA can integrate into the plant genome. The VirE2 proteins that coat the T-strand have to be removed and, the DNA duplicated to a double strand form needs to find a DNA break for integration. It has been published that VirF induced the degradation of both VIP1 and VirE2 when co-expressed in yeast and *in planta* (Tzfira *et al*., 2004).Therefore, it has been proposed that VirF is involved in the decoating of the T-complex. If this is an essential step in T-DNA transformation, it is difficult to understand why VirF is not an essential gene. A recent study revealed an *Agrobacterium*-induced *Arabidopis* F-box protein, designated VBF1 for VIP1-binding F-box protein, which performs a similar function as VirF, i.e. binding and destabilization of VirE2 and VIP1 (Zaltsman *et al*, 2010). Both VirF and VBF1 contribute to ubiquitin-mediated substrate proteolysis and promote genetic transformation.

The VirD5 protein is not required for tumorigenesis. VirD5 is predicted to contain four potential eukaryotic nuclear localization signals and is transported into the plant cell nucleus (X Zhang, personal communication). It was published recently that VirD5 counteracts host induced degradation of VirF by physical association with VirF (Magori and Citovsky, 2011).

8 *Agrobacterium* and plant defense responses

As a plant pathogen, *Agrobacterium* has PAMPs (pathogen associated molecular patterns) by which it elicits plant defense responses during infection (Ditt *et al*., 2006). Infiltrating tobacco leaves with *A. tumefaciens* induces the tobacco pathogenesis-related 1 (*PR-1*) gene and subsequently confers resistance to tobacco mosaic virus. Chlorosis, loss of chloroplast encoded rRNAs, and microRNA393

are also induced by *A. tumefaciens,* which contribute to antibacterial resistance (Pruss *et al*., 2008). Mutation of the *Arabidopsis* EF-Tu receptor called EFR (elongation factor thermo unstable receptor) results in enhanced susceptibility to *Agrobacterium* , as shown by a higher efficiency of T-DNA transformation, which shows that EF-Tu of *Agrobacterium* acts as an elicitor (Zipfel *et al*., 2006). Nevertheless, defense signaling through plant MAPKs (mitogen activated protein kinases) cascades also promotes *Agrobacterium* mediated transformation. Phosphorylation of VIP1 by MAPK3 at its Ser^{79} activates its NLS and facilitates its own nuclear import and that of the T-complex (Djamei *et al*., 2007). It is thought that in the nucleus, VIP1 and VirE2 proteins are ubiquitinated under control of VirF for degradation by the 26S proteasome (Tzfira *et al*., 2004). VirF, therefore, provides a link between ubiquitination and plant defense during *Agrobacterium* mediated transformation.

9 Ubiquitin system

Ubiquitin (Ub) is a small protein consisting of 76 amino acid residues, which is highly conserved and universally expressed in all types of eukaryotic organisms (Vierstra, 2009). As a post translational modification, protein ubiquitination plays a key role in protein half-life, localization and dynamics, important for many intracellular processes such as apoptosis, the cell cycle, DNA repair and signal transduction (Hua and Vierstra, 2011). The most studied function of ubiquitin is targeted protein degradation through the 26S proteasome.

Protein ubiquitination is composed of three consecutive steps: Ub-activation (E1s), Ub-conjugation (E2s) and Ub-ligation (E3). In the first step, Ub is activated by an E1 Ub activation enzyme which forms a thioester bond between the C-terminal carboxyl group of Ubiquitin and a cysteine residue of the E1 enzyme in an ATP dependent manner. Then, Ub is transferred from E1 to the active cysteine residue of an E2 ubiquitin conjugation enzyme. Finally, with the help of an E3 ubiquitin ligase, the ubiquitin is transferred from E2 to the target protein creating an iso-peptide bond between a lysine residue of the target protein and the C-terminal glycine of ubiquitin. In most cases, additional Ub molecules are added to the ubiquitinated protein resulting in a poly-ubiquitinated target protein (Hua and Vierstra, 2011; Welchman *et al*., 2005).

Polyubiquitinated proteins, especially those that are linked to the canonical lysine-48 and lysine-11 residues of Ub, are destined for degradation by the 26S proteasome. In contrast, proteins which are linked to the lysine-63 residue of Ub and mono-ubiquitinated proteins are not targeted to the 26S proteasome. Here, ubiquitination mainly has regulatory functions (de Bie and Ciechanover, 2011). Bioinformatic analysis of the *Arabidopsis g*enome sequence revealed that there are more than 1000 potential E3 ligases in *Arabidopsis*, making E3 ligases one of the largest protein families in this plant (Hua and Vierstra, 2011). The presence

of many different E3 ligases is crucial for the required substrate specificity of protein ubiquitination. The E3s have been generally classified into two groups: the HECT domain-containing E3 ligases (homology to the E6-associated protein carboxyl terminus) and the RING domain containing E3 ligases (really interesting new gene) (Smalle and Vierstra, 2004). Before transferring Ub to the target proteins, HECT E3 ligases form an intermediate by covalently binding to Ub, whereas RING domain E3 ligases promote directly transfer of Ub from E2 to the target proteins. RING-type E3s either consist of a single subunit (U-box domain proteins) or are multi-subunits E3-complexes referred to as Cullin-Ring ligases (CRLs) (Nakayama and Nakayama, 2006; Welchman *et al*., 2005). The RING finger is defined by the presence of the consensus sequence of C3HC4 (C, cysteine; H, histidine) or C3H2C3, which chelates two zink atoms. Single subunit E3s have a RING-finger E2-binding domain and substrate-binding domain on the same polypeptide. The Cullin-Ring ligases (CRLs), containing different protein-protein interaction modules, are the predominant class of E3s. The CRLs contain a common molecular architecture consisting of a cullin backbone, a RING-containing protein (RING-BOX, RBX-1) and a variety of adaptors which are responsible for recognition of the appropriate substrates for ubiquitylation (Vierstra, 2009).

CRLs affect numerous plant cellular processes including plant cell development, regulation of gene expression, cell cycle propagation, stress responses and pathogen defenses (Hua and Vierstra, 2011). In the *Arabidopsis* genome six genes have been found encoding cullins: CUL1, CUL2a/b, CUL3a/b, and CUL4. Each of the gene products assembles distinct types of CRLs according to their substrate adaptors. The largest and best characterized CRLs are SCF ubiquitin ligases (Hua and Vierstra, 2011). SCF complexes are composed of four major subunits: Cullin, Skp1 (S-phase kinase-associated protein), Rbx1 (Ring box protein) and the F-box protein. Cullin functions as a molecular scaffold in assembling the different subunits of the SCF complex by interacting with Skp1 at the amino terminus, with Rbx1 and a specific E2 Ubiquitin-Conjugating Enzyme (UBC) at the carboxyl terminus. *Arabidopsis* encodes more than 700 proteins with a (putative) F-box motif which are potential F-box subunits of SCF E3 ubiquitin ligase complexes and also in rice (*Oryza sativa*) more than 600 potential F-box proteins have been identified (Hua and Vierstra, 2011).

10 Plant defense and innate immunity

As sessile organisms, plants inevitably encounter a wide array of biotic challenges including plant pathogen and herbivore attacks during their life cycle.

Plant pathogenic microbes are classified into two or three groups based on the nutrients they use. Biotrophs require living plants as a source of energy, while necrotrophs kill their hosts and derive energy from the dead tissue. Hemibiotrophs, initially behave like biotrophs, but subsequently turn into

necrotrophs. In general, biotrophs are obligate parasites with a limited host range, whereas necrotrophs are non-obligate parasites with a broad host range (Glazebrook, 2005).

Plant pathogens trigger a defense response in the host plants called innate immunity. Pathogen-associated molecular patterns (PAMP) triggered immunity (PTI) is induced when the plant recognizes a pathogen. Pattern recognition receptors (PRRs) in the plant cell membrane recognize microbial components such as bacterial flagellin, lipopolysaccharides, peptidoglycan and fungal chitin (Nürnberger *et al*., 2004). The best-characterized plant PAMP recognition receptor is FLS2 (FLAGELLIN-SENSING2), a leucine rich repeat -receptor kinase, which recognizes and specifically binds to flg22, a 22 amino acid flagellin epitope. Upon activation defense responses are initiated after signal transduction by MAPK cascades and WRKY transcription factors (Asai *et al*., 2002; Gómez-Gómez and Boller, 2002; Zipfel *et al*., 2004). The bacterial elongation factor Tu (EF-Tu) has also been identified as an elicitor in plants and is recognized by the *Arabidopsis* leucine rich repeat-kinase called EFR(EF-TU RECEPTOR), which activates nearly identical plant defense responses (Zipfel *et al*., 2006).

However, (PAMP)-triggered immunity (PTI) can be suppressed by pathogens deploying secreted effector proteins which interfere with PTI and thereby contribute to virulence. For example, two effectors, named AvrPto and AvrPtoB, are delivered into plants by pathogenic *Pseudomonas syringae* strains using a type III secretion system, which successfully inhibit early steps in PTI, upstream of MAPKKK (He *et al*., 2006). Interestingly, the C-terminal domain of AvrPtoB has E3 ubiquitin ligase activity, suggesting it mimics host E3 ubiquitin ligases to inactivate plant defenses by degradation of host proteins (Janjusevic *et al*., 2006)

Effectors secreted by pathogens to counteract PTI can in turn be recognized by a different subset of receptors encoded by specific disease resistance (R) genes, which thereby trigger a second layer of defense, called effector-triggered immunity (ETI) (Nomura *et al*., 2011). R gene products fall into five distinct classes and most R proteins consist of a nucleotide binding domain (NB) and a leucine rich repeat (LRR) domain and are often referred to as NB-LRR proteins (Martin *et al*., 2003). When effectors of a pathogen are recognized by a cognate NB-LRR protein, ETI is induced. The effectors recognized by R genes are termed avirulence (Avr) proteins (Jones and Dangl, 2006).

11 Ubiquitination in plant disease resistance

Some plant-pathogen interactions are controlled by products of plant disease Resistance (*R*) genes against corresponding proteins of pathogen avirulence (*Avr*) genes, known as gene-for-gene resistance (Dodds and Rathjen, 2010;

Gururani *et al*., 2012; Biezen and Jones, 1998). *R-Avr* recognition results in rapid activation of defense responses including reactive oxygen intermediates (ROIs), the hypersensitive response (HR) and localized programmed cell death to inhibit pathogen growth within the plant cell.

The first clue about an involvement of E3-mediated proteolysis in plant defense came from the observation that the *Arabidopsis* R protein RPM1 (resistance to *Pseudomonas syringae* pv. *maculicola* 1) was rapidly degraded upon inoculation with *Pseudomonas. syringae* DC3000 (*avrRpm1 or avrB*), coincident with the onset of the hypersensitive response (Boyes *et al*., 1998). RPM1 is a plant R gene product containing a leucine zipper motif, a putative nucleotide binding site (NBS) and carboxyl-terminal leucine-rich repeat (LRR), which imparts resistance to *Pseudomonas syringae* DC3000 carrying either *avrRpm1* or *avrB* (Boyes *et al*., 1998). Two RING-finger type ubiquitin ligases named RIN2 and RIN3 (RPM1-interacting proteins) were found to interact with RPM1. The disappearance of RPM1 seems to be mediated by ubiquitination as it is blocked by treatment with a proteasome inhibitor (Kawasaki *et al*., 2005). However, the kinetics of the disappearance of RPM1 was not changed in a *rin2rin3* double-mutant after infection with DC3000 (*avrRpm1*)(Kawasaki *et al*., 2005), suggesting that degradation of RPM1 was neither mediated by RIN2 nor by RIN3.

The level of RPM1 was severely reduced in the *Arabidopsis rar1 (*Required for *Mla12* resistance 1) mutant, which suggests RAR1 may regulate RPM1 stability (Tornero *et al*., 2002). RAR1 encodes a 25 kD, cytosolic protein containing two 60-amino acid cysteine- and histidine-rich (CHORD) zinc binding domains that are conserved in all eukaryotes except yeast (Shirasu *et al*., 1999). The function of RAR1 required for R-gene-specified resistance against a variety of pathogens is conserved in different plant species (Bieri *et al*., 2004; Liu *et al*., 2002; Muskett *et al*., 2002; Tornero *et al*., 2002). RAR1 interacts with *Arabidopsis* orthologs of yeast SGT1 (for suppressor of the G2 allele of *skp1-4*), which associates with SKP1 and CUL1 to form an SCF ubiquitin ligase complex (Azevedo *et al*., 2002). Mutational analyses in *Arabidopsis* and silencing experiments in barley and *Nicotiana benthamiana* supports the widespread involvement of SGT1 in R-gene-triggered resistance against diverse pathogens (Austin *et al*., 2002; Azevedo *et al*., 2006; Disease and Team, 2003; Holt *et al*., 2005; Peart *et al*., 2002; Shirasu, 2003; Tör *et al*., 2002). Furthermore, the RAR1-SGT1 complex co-immunoprecipitates with CSN4 and CSN5, two COP9 (Constitutively Photomorphogenic 9) signalosome components associated with protein degradation by the 26S proteasome, suggesting a connection between R-gene mediated resistance pathways and ubiquitination by SCF E3 ubiquitin ligases (Azevedo *et al*., 2002). This hypothesis is supported by the observation that, the *N. benthamiana* SGT1 orthologue (NbSGT1) directly interacts with both NbRAR1 and NbSKP1, which is required for N gene-mediated defense to TMV (*Tobacco*

mosaic virus) (Liu *et al*., 2002). Besides, NbRAR1 and NbSGT1 associate with the COP9 signalosome and N-mediated resistance to TMV was compromised by silencing of NbRAR1, NbSGT1 or NbCOP9 signalosome components (Liu *et al*., 2002). Heat shock protein 90 (HSP90), a molecular chaperone, has been shown to interact with RAR1 and SGT1, and to be needed for resistance triggered by the R protein RPS2 against *P. syringae pv. tomato* DC3000 (*avrRpt2*) (Takahashi *et al*., 2003).

Cf-9 is another well characterized plant R gene, which confers resistance in tomato to *Cladosporium fulvum* by recognizing Avr9 (Rivas and Thomas, 2005). Further studies have revealed that numerous Avr9/Cf-9 Rapidly Elicited (ACRE) genes change their expression rapidly after elicitation of Cf-9 by Avr9 in tobacco cells (Durrant *et al*., 2000). Many of the ACRE genes encode transcription factors and protein kinases, but also ubiquitination pathway related proteins (Rowland *et al*., 2005). Three of these E3 ubiquitin ligase encoding genes, named *ACRE74*, *ACRE276* and *ACRE189*, were found to act as crucial positive regulators of plant R gene mediated disease resistance (Ewan *et al*., 2006; Tsitsigiannis *et al* 2006; Burg *et al*., 2008). *ACRE74*/*CMPG1* and *ACRE276* encode U-box type of E3 ubiquitin ligases, whereas *ACRE189*/*ACIF1* encodes an F-box protein. Silencing of *ACRE74*, *ACRE276* or *ACRE189 s*howed a reduced or prevented the hypersensitive response mediated by *Cf-9* genes (Ewan *et al*., 2006; Tsitsigiannis *et al*., 2006; Burg *et al*., 2008).

Genes responsible for the plant defense response have been identified by transcript profiling after addition of PAMPs such as bacterial flagellin (Navarro *et al*., 2004; Zipfel *et al*., 2004). Among the genes that were highly expressed after flg22 treatment were three plant U-box type E3 ubiquitin ligase encoding genes, PUB22, PUB23 and PUB24 (Felix *et al*., 1999). These three U-box type E3 ubiquitin ligases negatively regulate the PAMP-triggered defense response (Trujillo *et al*., 2008). A distinct enhancement of reactive oxygen species (ROS) production has been shown in the *pub22/23/24* triple mutant and activation of MPK3 is dramatically prolonged in the triple mutant (Trujillo *et al*., 2008). The gene *ATL2 ("Arabidopsis toxicos paralevadura") ,* encoding a putative RING-finger protein*,* was induced by the elicitor chitin (Salinas-Mondragón *et al*., 1999). Another member of the *ATL* gene family, named *ATL9,* was shown to play a positive role in the defense response to chitin and loss-of-function mutants of *ATL9* were more susceptible to the powdery mildew fungus *Erysiphe cichoracearum* (Ramonell *et al*., 2005).

12 Ubiquitination in Signal transduction during PTI and ETI

Three plant growth regulators, viz. salicylic acid, jasmonic acid and ethylene play an important role in the plant defense response. Defense against biotrophic pathogens is typically mediated by the salicylic acid signal transduction route. In

contrast, defense against necrotrophic paghogens is mediated by the jasmonic acid (JA) signal transduction pathway (Glazebrook, 2005). The SA and JA pathways affect each other by extensive cross-talk at different levels, while a third signal pathway triggered by ethylene (ET) also greatly contributes to the plant defense response.

Salicylic acid is pivotal in provoking the systemic acquired resistance (SAR) against biotrophic and hemibiotrophic pathogens (Grant and Lamb, 2006). Downstream the NPR1 protein (NON-EXPRESSOR OF PATHOGENESIS RELATED1) acts as a transcriptional coactivator, interacting with TGA2, which mediates expression of different PR genes (Fan and Dong, 2002). Mutations in *npr1* result in loss of resistance to pathogen infection, whereas overexpression of *npr1* shows increased induction of PR genes and consequential enhanced disease resistance in *Arabidopsis* and in rice (Chern *et al*., 2001; Cao *et al*., 1998). NPR1 contains a BTB/POZ (for Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) domain which associates with CUL3, forming a Cullin-RING ubiquitin ligase for degradation of specific substrates. BTB-containing proteins themselves might also be substrates for CUL3 based ubiquitin ligases. In the absence of pathogens, before binding to the target gene promoter, NPR1 is constantly degraded in the nucleus in a proteasome-dependent manner, preventing untimely activation of systemic acquired resistance. SA treatment leads to phosphorylation of NPR1 at residue Ser11 and Ser15, and stimulation of target gene expression before degradation (Spoel *et al*., 2009).

The F-box E3 ubiquitin ligase COI1 (CORONATINE INSENSITIVE1) protein was shown to play a role in the jasmonic acid signaling pathway (Xie *et al*., 1998). The *Arabidopsis coi1* mutant was identified by insensitivity to the bacterial toxin coronatine and showed enhanced susceptibility to infection by some fungal pathogens (Thomma *et al*., 1998). Recent studies showed that jasmonate ZIM domain (JAZ)-containing proteins function as negative regulators in the JA signal transduction pathway. In the absence of JA, JAZ proteins bind to the transcription factor MYC2 to inhibit defense gene expression, whereas in the presence of JA-isoleucine, the JAZ proteins are ubiquitinated by SCF^{COI1} for 26S proteasomal degradation, releasing MYC2 to activate defense gene expression (Chini *et al*., 2007).

The ethylene (ET) signaling pathway is induced by PAMPs and several pathogens. Ubiquitin-mediated proteolysis is also involved in ET signaling (Levin *et al*., 2004). EIN3 (Ethylene insensitive 3) is a transcription factor which can interact with ERF (ethylene response factor) genes, and thereby regulates gene expression in response to ethylene (Solano *et al*., 1998). In the absence of ethylene, EIN3 proteins are targeted for ubiquitination by F-box protein EBF1 or EBF2, whereas in the presence of ethylene, EIN3 is stabilized to mediate ethylene signaling (An

et al., 2010). The EIN3 transcription factor is under control of bifurcating and antagonistic MAPK cascades. MPK6 phosphorylates EIN3 at its threonine-174 residue, making it susceptible to degradation by SCF E3 ligases containing the F-box protein subunits EBF1 or EBF2 (EIN3 binding F-box). On the other hand, MPK6-independent phosphorylation of the threonine-592 residue inhibits EIN3 degradation, indicating the importance of ubiquitin-mediated proteolysis in the ET pathway (Yoo *et al*., 2008).

13 Effectors secreted by plant pathogens involved in ubiquitination

Plant pathogens deliver their virulence proteins into plant cells mostly either by a type three or four secretion system. Such virulence proteins may hijack or interfere with the host ubiquitin 26S/proteasome system (UPS) to facilitate their growth and cause plant disease.

The AvrPtoB protein of *Pseudomonas syringae* pv. *tomato*, which is secreted into plant cells by a T3SS shows at its C-terminus structural homology to RING/U-box type of E3 ubiquitin ligases (Janjusevic *et al*., 2006). The AvrPtoB protein ubiquitinates the plant kinase Fen, which is responsible for activating plant defense genes, and targets it for degradation by the 26S proteasome (Rosebrock *et al*., 2007). AvrPtoB also disrupts the PAMP signaling pathway by ubiquitination of the receptor-like kinase FLS2 (Göhre *et al*., 2008) and the LysM receptor kinase CERK1 (Gimenez-Ibanez *et al*., 2009).

Another *P. syringae* virulence protein HopM1 binds to the AtMIN7 protein and promotes degradation of AtMIN7 by the proteasome (Nomura *et al*., 2006). AtMIN7 is a member of the *Arabidopsis* adenosine diphosphate (ADP) ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) proteins and involved in the plant immune system (Nomura *et al*., 2006).

The GALA proteins secreted by *Ralstonia solanacearum*, a soil-borne phytopathogenic bacterium, all contain an F-box domain as well as a leucine-rich domain in the C-terminal region (Cunnac *et al*., 2004). The GALA7 protein, similar to *Agrobacterium* VirF, is a host specificity factor and is essential for disease on *Medicago truncatula*, indicating that the virulence function of GALA7 could be associated with ubiquitin mediated degradation of a plant protein (Angot *et al*., 2006).

Interestingly, microbes exploiting the host USP system are not restricted to plant pathogenic bacteria. Also some nitrogen fixing symbiotic bacteria use host UPS to facilitate nodulation. Recently, NopM, an effector of *Rhizobium* sp. strain NGR234, was found to function as a novel E3 ubiquitin ligase to promote nodulation on the host plant *Lablab purpureus* (Xin *et al*., 2012).

14 Outline of the thesis

AMT is widely used not only in plant molecular biology for elucidating the function of plant genes, but also for the construction of genetically modified plants resistant to pathogens or for the making of high yield crops. Besides, AMT has become a key tool for the genetic transformation of yeast and fungi for research purpose and for industrial biotechnology. Fully understanding of the mechanism of AMT is very relevant to improve the transformation efficiency and to improve the integration of the T-DNA at the desired chromosomal location.

During AMT, several virulence proteins such as VirD2, VirD5, VirE2, VirE3 and VirF are translocated from *Agrobacterium* into the host cells independent of the T-DNA (Vergunst *et al*., 2000). These translocated proteins greatly contribute to *Agrobacterium*'s virulence. The virulence protein VirF interacts with plant homologues of the yeast Skp1 protein, ASK1 and ASK2, suggesting that it forms an SCF complex that can ubiquitinate proteins destined for degradation. In support of this notion, it has been reported that both in yeast and *in planta* VirF is involved in the degradation of VIP1 and VirE2. However, direct evidence for VirF-mediated protein ubiquitination is still lacking. Another virulence protein, VirE3, was shown to mimic the function of the plant transcription factor VIP1 in facilitating T-DNA entry into the host nucleus. In addition, it binds to the plant-specific general transcriptional regulator pBrp. These observations suggest that VirE3 may affect the transcriptional machinery of the host cell. However, until now, direct evidence for a role of VirE3 in transcriptional regulation is lacking. In my research, I focused on the function of VirF in ubiquitination and degradation of host and *Agrobacterium* virulence proteins and on the function of VirE3 in plant transcriptional regulation.

VirF is not required for transformation of *S. cerevisae*, *Arabidopsis* and some other plant species. It has recently been published that in *Arabidopsis*, VBF, an *Arabidopsis* F-box protein, can mimic the function of VirF, thus explaining why VirF is not essential for the transformation of this plant species (Zaltsman *et al*., 2010). In chapter 2, it was investigated whether similarly, a yeast F-box protein could take over the function of VirF during AMT. To this end, 16 *S. cerevisiae* strains each lacking one of the genes encoding a (putative) F-box protein, were subjected to AMT using *Agrobacterium* strains deficient in *virF*. The experiments revealed that none of the yeast F-box proteins was required for transformation of *virF* mutants.

It has been reported that both in plants and in yeast VirF is involved in the degradation of VirE2 and VIP1 (Tzfira *et al*., 2004). In chapter 3, we analyzed whether VirE2 and VIP1 were degraded in a VirF-dependent way, whether VirF binds to the yeast SKP1 protein to form an SCF complex and whether potential yeast transcription factors with a similar function as the *Arabidopsis* VIP1 protein

can be degraded by expression of VirF. The results are in line with a minor role of VirF, if any, in targeted protein degradation in yeast.

During AMT VirE3 may bind to site in the host genome to locally activate the host cell's transcriptional machinery. However, expression of *virF* or *virE3* in yeast has a negligible effect on the genome-wide transcription profile (Chapter3). Because the plant is considered to be the natural host of *Agrobacterium*, in Chapter 4, the effect of expression of *virF* and *virE3* in *Arabidopsis* on the genome-wide transcription profile was investigated by using RNA-seq. Our results showed that expression of *virE3* had a considerable affect on the *Arabidopsis* transcriptome, in line with the proposed role of this virulence protein in transcription. Affected genes are enriched for genes involved signal transduction, stress response and response to biotic and abiotic stimuli. Compared to the expression of *virE3*, the effect of expression of *virF* was minimal.

Taken together, the function of *virF* in AMT seems to be quite limited in the model organisms *S. cerevisiae* and *Arabidopsis*. Our results do not support the hypothesis that VirF is involved in protein ubiquitination and degradation in yeast. In plants VirE3 may affect the transcriptional machinery to weaken the defense system and/or improve the transformation process.

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