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## **Agrobacterium infection : translocation of virulence proteins and role of VirF in host cells**

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# CHAPTER 1

## GENERAL INTRODUCTION

*Agrobacterium tumefaciens* is a Gram negative soil borne bacterial pathogen that causes crown gall disease in plants. Because of its capacity to genetically transform plant cells, *A. tumefaciens* is now used world wide as the most important vector system for production of transgenic plants. *Agrobacterium* delivers a so-called transfer DNA (T-DNA) into host cells. The process of T-DNA transfer is reminiscent of bacterial conjugation, and mediated by several bacterially encoded virulence proteins including a specialized protein complex spanning the bacterial envelope, the VirB/VirD4 type IV secretion system (T4SS) (Zambryski, 1988; Beijersbergen *et al.*, 1992; Pansegrau and Lanka, 1996). In addition, it delivers a set of effector proteins from the bacterium directly into host cells (Vergunst *et al.*, 2000; Schrammeijer *et al.*, 2003; Vergunst *et al.*, 2003; Vergunst *et al.*, 2005) where these contribute to bacterial virulence. Although much progress has been made to understand the process of T-DNA and protein delivery into host cells and T4SS biogenesis and structure (Christie and Cascales, 2005), only for several effectors the function is beginning to be understood. A clearer comprehension of the mechanisms by which this complex process leads to tumor formation is important to better adapt the vector system, and to exploit its potential optimally.

T4SSs are essential for the virulence of important pathogens, including the human pathogens *Helicobacter pylori*, *Bordetella pertussis*, *Legionella pneumophila*, and *Bartonella* spp. These human pathogens use this specialized secretion system to transfer only effector proteins into host cells where they subvert host biology in favor of the pathogen. Many bacterial effector proteins, including those transported by type III secretions systems, have been identified and seem to play a role in different host signaling pathways, such as GTPase signaling and ubiquitination processes, to interfere with the host's cell biology thus allowing colonization (Angot *et al.*, 2007; Hann and Rathjen, 2010; Hann, Gimenez-Ibanez and Rathjen, 2010; Hubber and Roy, 2010). One of the translocated effector proteins from *Agrobacterium*, VirF, carries an F-box domain by which it interacts with the *Arabidopsis* S-phase kinase (SKP1) associated protein (ASK1), member of the SKP1-Cullin-Fbox (SCF) E3 ubiquitin (Ub) ligase (Schrammeijer *et al.*, 2001). The SCF complex is involved in earmarking

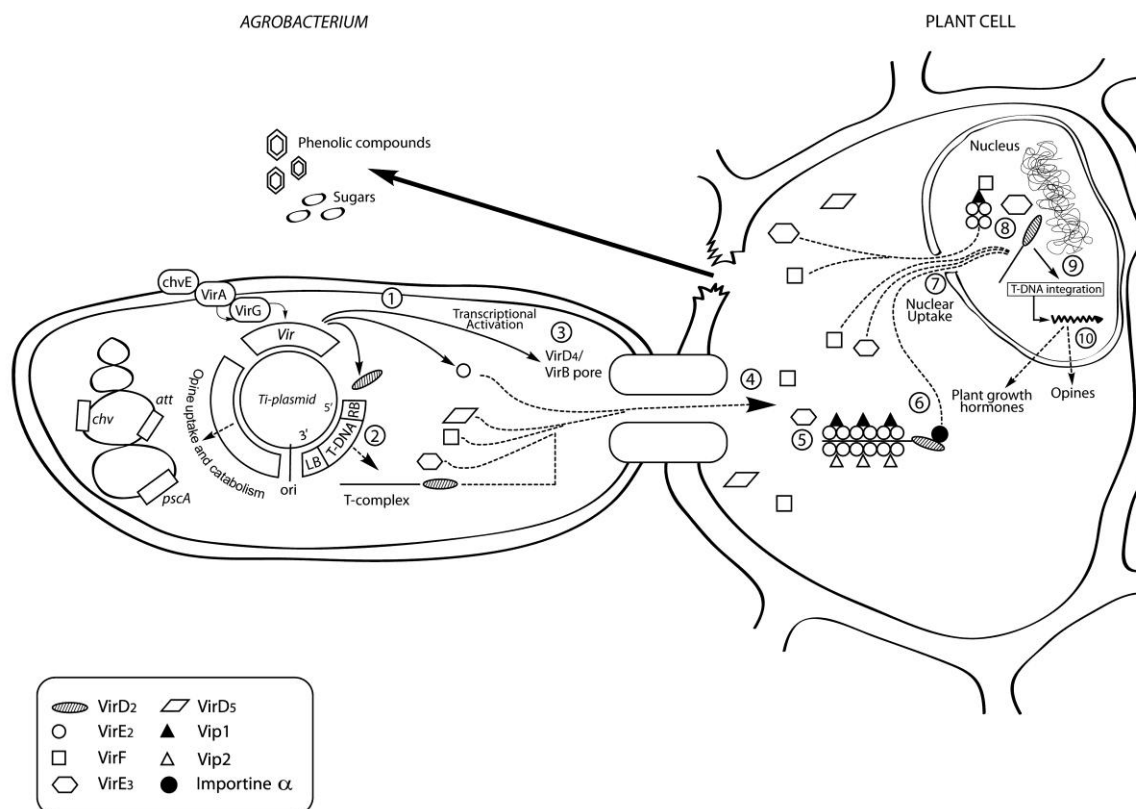
substrates by ubiquitination for targeted degradation by the proteasome in eukaryotic organisms (Hershko and Ciechanover, 1998), and therefore VirF was suggested to play a role in ubiquitination processes. The present project aimed to identify possible host target proteins of VirF, and study their interaction with VirF in detail using *Arabidopsis thaliana* and *Saccharomyces cerevisiae* as model organisms. In this chapter I first describe the *A. tumefaciens* transformation process, followed by a description of the state of the art of the role of ubiquitination in host cell homeostasis and host-pathogen interactions.

## **1. *Agrobacterium tumefaciens* AND PLANT TRANSFORMATION**

### **1.1 General biology and morphology**

*A. tumefaciens* is a soil-borne bacterium belonging to the family of *Rhizobiaceae* of the  $\alpha$ -proteobacteria which includes various nitrogen-fixing plant symbionts collectively called rhizobia (*Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium*, and *Bradyrhizobium*). *A. tumefaciens* together with other species (*A. rubi*, *A. vitis*, *A. rhizogenes* and *A. radiobacter*) are known as agrobacteria, which with the exception of the last one, are plant pathogens that cause crown gall tumors and other neoplasias on a wide variety of plants (Binns and Costantino, 1998; reviewed by Brencic and Winans, 2005). Some authors have proposed that all these *Agrobacterium* species should be relocated inside the genus *Rhizobium* and that the genus *Agrobacterium* might be abandoned (Young *et al.*, 2003). Farrand *et al.* (2003) have countered this proposal by demonstrating that the *Agrobacterium* species are sufficiently different from the rhizobia to warrant maintenance of the *Agrobacterium* genus name. *A. tumefaciens* (C58) contains a linear (2,07 mb), and a circular chromosome (2,84 mb) that encode for all basic structural and functional genes (Goodner *et al.*, 2001; Wood *et al.*, 2001), including the chromosomal virulence genes (*chv*) (Lippincott and Lippincott, 1969; Douglas *et al.*, 1982). Several chromosomal genes such as *chvA*, *chvB*, *chvE*, *chvI* and *pscA* (*exoC*) are important for stable maintenance in the plant and attachment to plant cells. Mutation of these genes leads to avirulent strains (Zhu *et al.*, 2000). In addition to the chromosomes, the bacterium also carries large circular plasmids: a so-called 'cryptic' plasmid (pAtC58, 543 kb) Rosenberg and Huguet, 1984; Goodner *et al.*, 2001) and in virulent strains the tumor inducing (Ti) plasmid (pTiC58) (Zaenen *et al.*, 1974; Van Larebeke *et al.*, 1975; Watson *et al.*, 1975). All this together represents a 5,67 megabase genome with a total of 5419 predicted coding genes (Wood *et al.*, 2001). The Ti plasmid is an independent replication unit of around 200Kb that carries the DNA segment that is transferred to host cells during tumorigenesis. The T-DNA contains oncogenes that mediate the conversion of normal plant cells into tumour cells. Some of the T-DNA oncogenes encode enzymes involved in the production of plant growth regulators, more specifically the auxin indole acetic acid and a cytokinin. Their expression leads to the local overproduction of other plant hormones such as jasmonate, ethylene and abscisic acid as well and this leads to tumor formation and tumor vascularization (Ullrich and Aloni, 2000). The T-DNA also harbours genes involved in the production of tumour specific metabolites called opines that are formed by condensation of an amino acid, either with a keto acid or a sugar. Opines can be used as a source of nitrogen

and energy by *Agrobacterium*. The genes for opine catabolism are located on the Ti-plasmid in a region adjacent to the genes required for conjugation (*tra* and *trb* genes) and vegetative replication (*rep*), which are highly conserved between different types of Ti plasmids.



**Figure 1. An overview of infection and T-DNA transfer to plant cells by *Agrobacterium*.** After a plant cell is wounded and liberates phenolic compounds and sugars, a sequence of events follows that initiates with attachment of bacteria to host cell and the activation of the *vir* genes. T-DNA transfer is brought about in several steps as depicted above: **1)** Biosynthesis of Vir proteins; **2)** VirD2 T-DNA lower strand nicking with assistance of VirD1 and VirC1, eventually leading to the formation of the T-strand with VirD2 covalently attached (T-complex); **3)** Assembly of the VirD4/VirB T4SS; **4)** Translocation into the host cell of the T-complex (piloted by VirD2) and the Vir proteins VirD5, VirE2, VirE3 and VirF after recognition of their protein translocation signal by the coupling protein VirD4; **5)** Coating of the T-strand inside the host cell by VirE2 dimers to unfold the T-strand and protect it against host nucleases **6)** Interaction of VirE2 with the Vip1, Vip2 proteins and plant cytoplasmic receptors (importin  $\alpha$ ) that recognize the nuclear localization signals both in VirE2 and VirD2; **7)** Nuclear uptake of the T-complex; **8)** T-DNA uncoating from VirE2 via interaction of Vip1 with VirF. **9)** T-DNA integration into the plant genome by non-homologous recombination; **10)** Expression of T-DNA genes leading to production of plant hormones and opines required for *Agrobacterium* growth.

In addition, the Ti-plasmid contains the *vir* region encoding approximately 25 virulence proteins required for the proper transfer and integration of the T-DNA into the host cell (reviewed by White and Winans, 2007). The *vir* genes are organized in a number of operons. Operons *virA*, *virB*, *virC*, *virD*, *virE*, and *virG* encode for genes that are essential for pathogenesis (Stachel and Nester, 1986; Stachel and Zambryski, 1986; Toro *et al.*, 1989; reviewed by Brencic and Winans, 2005), while mutations in *virD5*, *virE3*, *virF*, *virH*, *virJ*, *virK*, *virL*, *virM*, *virP* and *virR* genes may lead to a restriction in the plant host range or to an

attenuation of tumorigenicity (Kalogeraki and Winans, 1998; Kalogeraki *et al.*, 2000; Zhu *et al.*, 2000; Pantoja *et al.*, 2002). Chemical recognition of the host by *A. tumefaciens* is the starting point that precedes activation of virulence gene expression. Upon plant wounding, phenolic compounds such as acetosyringone [AS] and hydroxy-acetosyringone [OH-AS] are liberated. These trigger the autophosphorylation of the constitutively expressed VirA receptor protein (Jin *et al.*, 1990a). A monosaccharide-binding protein, ChvE, which recognizes sugars in the plant sap (Shimoda *et al.*, 1993), interacts with the periplasmic domain of VirA and enhances its sensitivity and maximal response to phenolic compounds (Gao and Lynn, 2005). Subsequently, VirA phosphorylates the responsive regulator VirG (Jin *et al.*, 1990b), which binds then to a *cis*-element upstream of the different *vir* promoters known as the *vir* box. This leads to activation of the *vir* regulon and expression of the virulence proteins (Jin *et al.*, 1990abc) (Figure 1).

Under *in vitro* conditions, induction of *vir* genes is possible in the presence of acetosyringone [AS], a low pH of the medium, as in plant sap, and temperature of 28°C or below (Turk *et al.*, 1991). Using such laboratory conditions the virulence system of *A. tumefaciens* can mediate interkingdom DNA transfer not only to plant cells, but also to yeasts (Bundock *et al.*, 1995) and fungi (De Groot *et al.*, 1998), being used in the last number of years specially as standard tool for transformation for relevant fungal species (Michielse *et al.*, 2005; Kemppainen and Pardo, 2011). Single reports have appeared indicating that transfer could even be possible to cells of sea urchins (Bulgakov *et al.*, 2006) and some mammalian cells as well (Lacroix *et al.*, 2006).

## **1.2 T-DNA transfer and integration process**

The process of *Agrobacterium* infection involves the attachment of the bacteria to host cells and the subsequent introduction of its T-DNA. This process is assisted by several of the Ti encoded virulence proteins, which play a role in forming the translocation channel known as the type IV secretion system (T4SS), as well as in the mobilization of T-DNA and effectors within the plant cell via this channel. The outcome of this process is the integration of T-DNA into the host genome that eventually allows the expression of the bacterial T-DNA genes in the plant cells in favor of *Agrobacterium* growth.

### **1.2.1 T-DNA transfer**

The T-DNA transfer apparatus is a T4SS that likely evolved from the DNA conjugation systems used by bacteria for horizontal DNA transfer. The DNA molecules that are transferred characteristically carry an origin of transfer (*oriT*), a short DNA sequence that is essential for transfer and which is nicked by the conjugative relaxase to start the formation of the single stranded DNA copy (T-strand) which is actually transferred. Functionally, the conjugal transfer machinery can be seen as to consist of three parts. The first, the relaxosome complex, is formed by an *oriT*, and the DNA transfer and replication (Dtr) proteins: a relaxase and one or more accessory proteins. Because of its specificity for each plasmid system, this structure is also known as the *selector* and is considered to produce the substrate for translocation. The second, the transmembranal *conduit*, is a multiprotein

complex composed of around 10 different proteins belonging to the T4SS family that spans both the inner and the outer membrane; and the third, the *coupling protein* (CP), brings the *selector* to the *conduit*. Additionally, it has been proposed that the CP actively pumps the T-strand from the donor into the recipient cell (Llosa and de la Cruz, 2005).

The T-DNA in the Ti plasmid is surrounded by border repeats of 25 bp that are equivalent to the *oriT* and required for its mobilization into plant cells. In *Agrobacterium* the T-strand corresponds to the bottom strand of the T-DNA region such that its 5' and 3' ends map to the right and left T-DNA border repeats. VirD2 is the relaxase (Pansegrau and Lanka, 1996), that recognizes and together with VirD1 cleaves the lower strand of the 25 bp T-DNA border repeat sequences. Subsequently, by a strand displacement mechanism, a single stranded (ss) linear DNA copy of the bottom strand (T-strand) is released (reviewed by Mysore *et al.*, 1998). The VirD2 protein remains covalently attached to the 5' end of the T-strand thus forming a T-complex, and pilots the T-strand into the host cell. The products of the *virC* locus (VirC1 and VirC2) bind to a sequence adjacent to the right border called overdrive and thus act as enhancers of the nicking reaction and of T-strand formation (Toro *et al.*, 1988; van Haaren *et al.*, 1987; De Vos and Zambryski, 1989; Lu *et al.*, 2009). The VirC1 protein has an additional role in the recruitment of the T-complex to the cell poles, where the VirB/D4 T4SS machine assembles (Atmakuri *et al.* 2007). Recently, three *Agrobacterium* VirD2-binding proteins (VBPs) with a putative nucleotidyltransferase motif were reported. Their mutation highly affects tumor development. One of the encoding genes, *vbp1*, is located in the cryptic pAtC58 plasmid, while the two other are part of the linear chromosome. All three *vbp* genes are located close to genes that are highly similar to conjugative transfer genes. Only the mutation of all three genes leads to a highly attenuated virulence, indicating that the encoded proteins play an important role in the transfer of the T-complex to plant cells (Guo *et al.*, 2007).

Currently, for plant transformation the so-called binary vector system is used by which any foreign DNA sequences can be conveniently inserted into a multiple cloning site that is located between border repeats in an artificial T-region. Subsequently, this plasmid is introduced into an *Agrobacterium* strain harboring a Ti plasmid (helper) that contains an intact *vir* region, but lacks the T-region (Bevan, 1984; De Framond *et al.*, 1983; Hoekema *et al.*, 1983; An *et al.*, 1985). The virulence functions encoded by the helper Ti-plasmid mediate the translocation of the T-DNA segment from the binary plasmid into a recipient cell, where it becomes integrated into the genome.

### 1.2.2 Structure of the transport system

Secretion systems related to bacterial conjugation are referred to as the T4SS, which have been classified into four subgroups according to their specific function: effector molecule translocation into host target cells, conjugation of chromosomal and plasmid DNA; DNA uptake and transformation and DNA release into the extracellular milieu (reviewed by Backert and Meyer, 2006). The main structural and functional characteristics of type IV machinery are conserved between the different T4SS classes. By reviewing the structural properties of the T4SS used by different bacteria, Franzes, Christie and Waksman (2009)

reduce this classification to three types: (a) T4SS involved in the mediation of conjugal transfer of plasmid and transposon DNA between bacteria, (b) those used by bacteria such as *Helicobacter pylori* and *Neisseria gonorrhoeae* for uptake of DNA from and release of DNA into the extracellular milieu and (c) T4SS of animal/human bacterial pathogens such as *Bordetella pertussis* and *Legionella pneumophila* required for translocation of effector virulence proteins into eukaryotic hosts. However, the virulence system of *A. tumefaciens* embraces a T4SS that is involved both in the transfer of effector proteins as well as DNA molecules into eukaryotic cells.

The fact that effector virulence proteins can be translocated in absence of T-DNA using the T4SS (Vergunst *et al.*, 2000; Schrammeijer *et al.*, 2003; Vergunst *et al.*, 2005) suggests that DNA transfer systems have evolved from protein transfer systems, and that it is actually the pilot protein linked to the DNA, which is recruited and recognized by the T4SS (van Kregten *et al.*, 2009). Eleven proteins encoded by the *virB* operon constitute the *Agrobacterium* TFSS (Zupan and Zambryski, 1997; Hansen and Chilton, 1999; Zhu *et al.*, 2000; Hamilton *et al.*, 2000). This T4SS forms a multisubunit cell envelope-spanning structure required for substrate transfer and also elaborates a T-pilus, which is important but not essential for intimate contact (attachment) between the *Agrobacterium* cell and the host plant cell (Lai *et al.*, 2000). According to protein localization and function, a set of subcomplexes can be defined. **First**, the cytoplasmic ATPases located towards the inner membrane, VirD4, VirB4 and VirB11, which form hexameric ring structures *in vitro* that may form pores in the inner membrane *in vivo* and which may play an important direct role in substrate translocation as a pump and/or in powering the translocation machinery; **second**, the translocation pore complex, mainly formed by a core located along the inner membrane (IM), periplasm and outer membrane (OM) composed of 14 subunits each of the VirB7, VirB9 and VirB10 proteins, (reviewed by Wallden, Rivera-Calzada and Waksman, 2010; Waksman and Fronzes, 2010) and which are in contact with the inner membrane basic pore composed of the VirB6 and VirB8 proteins and **third**, the extracellular T-pilus, which is predominantly composed of VirB2, but which also contains VirB5 and possibly interacts with the host VirB2-interacting proteins BTI1, BTI2 and BTI3 and AtRAB8, a membrane-associated GTPase (Hwang and Gelvin, 2004). At the site of T4SS assembly the VirB1 protein is required for localized lysis of the murein layer between the outer membrane and periplasm (Ward *et al.*, 2002; Zahrl *et al.*, 2005). All T4SS that mobilize DNA-protein complexes, and many that mobilize only proteins, have a NTPase that serves as a coupling protein (CP) that specifically recruits substrates to the transport complex and also interacts with certain subunits of the transport complex (Cascales and Christie, 2004a, Llosa *et al.*, 2003). Recognition of the T-strand by the coupling protein VirD4 requires the catalytically active VirD2 relaxase, indicating that the T4SS CP binds the DNA only by mediation of the relaxase (reviewed by Alvarez-Martínez and Christie, 2009).

Use of transferred-DNA immunoprecipitation assays (TriP) allowed Cascales and Christie (2004b) to propose a sequence of interactions during the transfer of the T-strand through the VirB complex crossing of the inner membrane, periplasm/peptidoglycan wall and outer membrane, as well as the plant host cell wall. The inner membrane associated

components interact with the T-strand first, followed by periplasmic and outer membrane-associated components. Initially, the T-strand interacts with VirD4, followed by binding to VirB11, then to VirB6 and VirB8, and finally interacts with VirB2 and VirB9. The interaction of the T-strand with VirD4 does not require any VirB proteins, whereas binding to VirB11 requires VirD4 and VirB7 and binding to VirB6 and VirB8 requires VirB4. The final step, the interaction of the T-strand with VirB9 and VirB2, may require all the VirB proteins. In addition, ATP hydrolysis by one or all of the NTPases may be required for the substrate translocation across the inner membrane or movement to and through the periplasm. The VirD4, VirB4, and VirB11 proteins each have a nucleoside triphosphate binding site (Walker A motif). Mutations in these motifs do not affect T-strand interaction with VirD4 and VirB11 (Atmakuri *et al.*, 2004), but they do arrest substrate transfer to VirB6 and VirB8. These studies have shown the possible way of movement of the T-strand sequentially along VirD4, VirB11, VirB6, VirB8, VirB9 and VirB2; although the other components of the VirB/D4 channel apparently do not interact directly with the T-strand, they are also essential for its transfer (reviewed by Franzes, Christie and Waksman, 2009).

### **1.2.3 *Agrobacterium* effector proteins and their role in T-DNA transfer and integration into the host cell**

In addition to the transfer of the T-DNA through the bacterial inner and outer membrane into the host cells, the T4SS is also required for the translocation of the virulence effector proteins VirD2, VirD5, VirE2, VirE3, and VirF. Such transfer occurs independently of DNA transfer as shown in the Cre recombinase reporter assay for translocation (CRAFT), which was developed in our lab to investigate this (Vergunst *et al.*, 2000; Schrammeijer *et al.*, 2003; Vergunst *et al.*, 2003; Vergunst *et al.*, 2005). In this assay fusions of VirE2 and VirF to the Cre enzyme were expressed in the bacterium to visualize transport of these effectors into the host cell genome by a Cre-mediated recombination event that conferred kanamycin resistance in plants or an ability of yeast cells to grow in presence of fluoro-orotic acid (FOA). Interestingly, only N-terminal fusions were translocated, indicating that blocking of the C-terminal region had a negative influence on the recognition by the transport system. Additional virulence proteins such as VirD2, VirD5 and VirE3 were similarly translocated into host cells using a C-terminal transport signal (Schrammeijer *et al.*, 2003; Vergunst *et al.*, 2005). The 30-50 C-terminal amino acids (aa) of VirF, VirE2 and VirE3 were found to be essential to mediate transport to plant cells (Vergunst *et al.*, 2000; 2003). Further detailed mutational analysis of the translocation signal of VirF revealed the importance of positively charged amino acids in this region: by comparison of translocated substrates a positively charged region with the consensus sequence (R-X<sub>(7)</sub>-R-X-R-X-R; Vergunst *et al.*, 2005) was identified. Importantly, other T4SSs, such as the Legionella Dot/icm system (Roy *et al.*, 2005) have also shown the use of C-terminal transport signals (reviewed by McCullen and Binns, 2006).

Protein translocation experiments revealed that besides the VirB proteins the presence of VirD4 is required for the transport of both DNA as well as the effectors VirE2 and VirF (Vergunst *et al.*, 2000), VirE3 (Schrammeijer *et al.*, 2003), VirD5 and VirD2 (Vergunst *et*



*al.*, 2005). The coupling protein, VirD4 is thought to be responsible for the recruitment of substrates for translocation through the T4SS (Christie, 1997; Lai *et al.*, 2000) as shown by Cascales and Christie (2004b) for the T-complex by use of transferred-DNA immunoprecipitation assays (TrIP). In addition, *in vivo* experiments have revealed interaction between VirE2 and VirD4 and that truncation of the C-terminus of VirE2 prevents this interaction, what underscores that the C-terminal signal identified in the *A. tumefaciens* substrates plays a role in their recognition by the translocation apparatus (Atmakuri *et al.*, 2003).

The VirE2 protein is the most important effector protein. It binds cooperatively to the T-strand and protects it against nucleolytic attack inside the host cell. This association stabilizes the nucleoprotein complex to promote its transfer into the nucleus (Ziemienowicz *et al.*, 2001). Joining of VirE2 subunits to the T-strand can take place in the plant cell as indicated by 'extracellular complementation' experiments. It cannot be excluded, however, that such T-complex formation also can occur inside the VirB/D4 transport channel, with simultaneous release of its chaperone protein VirE1 (Frenkiel-Krispin *et al.*, 2006). The interaction with VirE1 avoids self-aggregation of VirE2 keeping it in a proper state for translocation. This may also prevent its premature binding to the T-strand or to other proteins inside the bacterial cell facilitating the transport through the TFSS (Citovsky *et al.*, 1989; Deng *et al.*, 1999; Sundberg and Ream, 1999; Zhou and Christie, 1999; Sundberg *et al.*, 1996). VirE1 is not necessary for recognition of VirE2 as a translocation substrate by the TFSS; the C-terminus of VirE2 can mediate translocation of Cre in the absence of VirE1 (Vergunst *et al.*, 2003). The crystal structure of the VirE2-VirE1 complex revealed that VirE2 is composed of two independent domains that are joined by a flexible linker. By interaction with VirE1 the two domains of VirE2 are locked in an orientation that prevents VirE2-VirE2 interaction and aggregation and binding to ssDNA (Dym *et al.*, 2008).

The VirE3 protein is another T4SS translocated effector (Schrammeijer *et al.*, 2003). It is transported into the host cell nucleus where it may induce the expression of genes needed for tumor development (García-Rodríguez, Schrammeijer and Hooykaas, 2006). Similarly, the VirF protein is translocated by the T4SS into host cells (Vergunst *et al.*, 2000; Schrammeijer *et al.*, 2003), where it may play a role in ubiquitin-mediated proteolysis (Schrammeijer *et al.*, 2001). The fact that *A. tumefaciens* double *virF/virE3* mutants are more strongly attenuated in virulence than the single mutants, suggests that VirE3 and VirF have a partly redundant function during bacterial infection (Schrammeijer, 2001; García-Rodríguez, Schrammeijer and Hooykaas, 2006). The function of VirD5 is so far completely unknown.

### **1.3. Host factors involved in *Agrobacterium* infection**

The identification of the host proteins that are targeted by bacterial infection is of great relevance to understand the full biology of pathological interactions. The increasing use of *Agrobacterium* as a natural biological vector for the transfer of genes to eukaryotic organisms gives an extra impulse to research aimed to understand which and how host factors are involved in infection and processes during *Agrobacterium* tumor formation such as attachment, intracellular T-DNA transport and T-DNA integration.

When *Agrobacterium* enters in contact with the plant host, certain compounds that are required for the initial attachment and chemotaxis to the plant cells are already present. However, little information is available in relation to the nature and function of the factors that *Agrobacterium* utilizes as specific receptors on the host cell surface and/or cell wall for attachment and adhesion. Among these putative receptors and host proteins are a vitronectin-like protein (Wagner and Matthysse, 1992), a rhicadhesin-binding protein (Swart *et al.*, 1994), a cellulose synthase-like gene (Zhu *et al.*, 2003a), which act as cell adhesion proteins or attachment factors, and several VirB2-interacting proteins (BTIs) (Hwang and Gelvin, 2004). How and when these proteins play a role in the transformation process is still largely unknown (reviewed by Citovsky *et al.*, 2007). In maize, which is recalcitrant to *Agrobacterium* transformation, a naturally occurring compound is present known as 2-hydroxy-4,7-dimethoxybenzoxazin-3-one (DIMBOA). This was shown to be a potent inhibitor of the *vir* genes (Sahi *et al.*, 1990), and therefore, of the transfer of the T-DNA into the host plant. This may assist the plant in its local defense against agrobacteria.

### 1.3.1. Fate of the T- complex in the host cell

T-DNA processing and transfer to the host is mediated by the bacterial virulence proteins without apparent influence of host factors. In advanced stages of infection, once the T-complex has been translocated into the host cytoplasm, host factors seem to play an important role in facilitating the translocation of the T-complex into the nucleus and its integration into the host genome. Uptake of the T-complex in the nucleus is mediated by the interaction of both VirD2 and VirE2 with host proteins (Tzfira *et al.*, 2001). Nuclear entry occurs via an importin-dependent pathway, which depends on the presence of nuclear localization sequences in VirD2 and in VIP1, the binding partner of VirE2 (reviewed by Lacroix *et al.*, 2008).

VirD2 interacts with importin- $\alpha$  (Ballas and Citovsky, 1997; Bako *et al.*, 2003), which guarantees uptake into the nucleus, and with cyclophilins (Deng *et al.*, 1998), which may assist VirD2 in keeping an active conformation. In addition, two other plant proteins have been shown to have an interaction with VirD2: a cyclin dependent kinase-activating protein (CAK2Ms) that phosphorylates VirD2, and a TATA box-binding protein (TBP) (Bako *et al.*, 2003). Phosphorylation may promote nuclear entry, while the interaction with TBP may affect the efficiency of integration into the genome. By the cooperative binding of the VirE2 protein to the T-strand this is protected from nucleases and forms a long, thin thread without knots which is facilitating import into the host cell nucleus (Lacroix and Citovsky, 2009). Once inside the plant cell, the VirE2 protein also interacts with VirE2 interacting proteins 1 and 2 (VIP1 and VIP2). Possibly, VIP1, VIP2, and VirE2 function in a multiprotein complex that facilitates T-DNA nuclear import, intranuclear transport of the T-complex and integration of T-DNA into the host genome. Studies in mammalian cells have shown that many DNA viruses use dynein motor proteins and the host microtubule network as a transport system through the cytoplasm in their way towards the nucleus (reviewed in Henry *et al.*, 2006). It has been suggested that the *Agrobacterium* T-complex is transported through the host cell cytoplasm by a similar cellular mechanism (Salman *et al.*, 2005; Tzfira, 2006), possibly by a dynein-like

*Arabidopsis* protein (DLC3) that associates with the plant microtubules and may function as a link between VIP1–VirE2–T-DNA complexes and the microtubule track system (Citovsky *et al.*, 2007). It is believed that via interaction with core histones, VIP1 may bring the T-complex to the host chromatin (Lacroix, Loyter and Citovsky, 2008). The VIP2 protein encodes a NOT (for negative on TATA-less) domain-containing protein that interacts with VirE2. Knockout *vip2* plants are defective in stable T-DNA transformation showing less integrated T-DNAs than wild type plants (Anand *et al.*, 2007). This suggests that VIP2 is required for *Agrobacterium*-mediated plant transformation by facilitating T-DNA integration into plant chromosomes.

It is not known exactly which proteins are involved in the integration of the T-DNA into the plant genome or whether this integrates as a single or double stranded DNA molecule. It has been reported that in *Saccharomyces cerevisiae* T-DNA integrates preferably by homologous recombination (HR), but in the absence of DNA homology, insertion into the genome occurs by non-homologous recombination (NHR) as this occurs in plant cells (Bundock *et al.*, 1996). The host proteins involved in T-DNA integration were identified in yeast: non-homologous T-DNA integration was found to depend on the proteins involved in non-homologous-end joining (NHEJ), viz. the heterodimer Ku70/Ku80 and the Lig4 (van Attikum *et al.*, 2001). Integration by HR was entirely dependent on Rad52 (van Attikum *et al.*, 2003a). If both NHEJ and HR had been inactivated, T-DNA integration was no longer possible. It was also demonstrated in our laboratory that in other yeasts and fungi non-homologous T-DNA integration also relied on the NHEJ complex and that by its inactivation the T-DNA could very effectively be directed to a process of integration by HR (gene targeting) (Hooykaas, van Attikum and Bundock, 2000; Kooistra *et al.*, 2004). In plants, however, backup systems can mediate non-homologous integration even after inactivation of the classical pathway of NHEJ (van Attikum *et al.*, 2003b; Jia, 2011).

### **1.3.2. *Agrobacterium* and plant defense response**

The presence of *Agrobacterium* also elicits defense responses in the plant as can be seen from the up and down regulation of a series of defense genes at early and later stages of the infection process (Ditt *et al.*, 2006). When *Agrobacterium* is infiltrated into plant leaves, it induces a series of local responses consisting of induction of pathogenesis-related (PR) gene expression and resistance to subsequent infection with tobacco mosaic virus, together with chlorosis and loss of chloroplast rRNAs and inhibition of leaf expansion. Also the levels of microRNA miR393 are increased. This microRNA represses auxin signalling and contributes to antibacterial resistance (Pruss, Nester and Vance, 2008). Such defense responses are provoked when a plant senses a pathogen by its pathogen-associated molecular patterns (PAMPs). The receptor for one of these PAMPs, the bacterial translation elongation factor EF-Tu was identified in *Arabidopsis thaliana*. Mutants defective in this receptor showed enhanced susceptibility for infection by *Agrobacterium*, which has an EF-Tu that is fully active as an elicitor (Zipfel *et al.*, 2006). PAMPs are also present on the surface of *Agrobacterium*: the peptidoglycan and muropeptides from *Agrobacterium* were found to elicit an innate immunity, although similar compounds from the aggressive pathogen

*Xanthomonas campestris* were significantly more active (Erbs *et al.*, 2008). This is in line with the requirement of host cell viability during *Agrobacterium* infection. Studies by Djamei *et al.* (2007) showed that a MAPK kinase phosphorylation cascade, which is activated as a defense response to infection, activates VIP1 to migrate into the nucleus where it may promote transcription of pathogenesis related genes (PR). On its way to the nucleus, VIP1 may bind to the T-complex through association with VirE2 and thus help to bring the T-complex into the nucleus. Apparently, *Agrobacterium* uses this defense signalling of the cell to deliver its T-complex into the host-cell nucleus.

This shows that *Agrobacterium* has evolved into an organism that is able to profit from the defense machinery of its host in favor of its settlement. During the final stage of *Agrobacterium* infection in which integration of the T-DNA inside the host genome is required, both VirE2 and VIP1 must be removed to expose the T-DNA for integration. Studies by Tzfira *et al.* (2004) suggested that due to its interaction with VIP1, VirF may assist in this process as an F-box protein. During the present research work we step into the study of VirF inside the host cell and its interactions with host proteins looking at the possible ways of how it may work as part of an SCF complex. Recent studies have revealed that a host defense-related VIP1-binding F-box protein (VBF), which is induced by *Agrobacterium* infection, may be also involved in this process using a similar mechanism as VirF (Zaltsman *et al.*, 2010). This indicates that *Agrobacterium* has evolved the necessary tools as a pathogen to exploit the host cell machinery in pro of its settlement, whereby the ubiquitin-proteasome system (UPS) is one of its targets.

## 2. UBIQUITIN MEDIATED PROTEOLYSIS

The half life of different proteins varies from as short as minutes to as long as several years, but as part of life, sooner or later proteins will have to be removed (after they have accomplished their function or after they have been damaged), and have to be recycled by the cell machinery. Biologically this is translated into protein degradation, which is for the cell as important as generative processes. The regulation of protein levels by protein destruction has been valued as a relevant step towards homeostasis and life progression (reviewed by Thompson *et al.*, 2008). Concurrent protein synthesis and proteolysis determine then the steady state levels of proteins in (eukaryotic) organisms. The discovery of the dynamic state of proteins was followed by the discovery of the lysosome, which until the 1970s was believed to be the only place where proteins were destroyed intracellularly. However, on basis of new experiments, soon after a concept developed that intracellular proteins are degraded via a non-lysosomal machinery as well. This culminated in the discovery of the ubiquitin-proteasome system at the end of the 1970s (Ciechanover, 2006).

For a protein to be destroyed specifically, first, it needs to be targeted by a highly specific protein-degradation system, which for proper recognition, unfolding and cleavage of this substrate, usually requires considerable levels of energy (Tanaka, Waxman and Goldberg, 1983; Hershko *et al.*, 1984). In eukaryotes most of the proteins are specifically degraded via the ubiquitin proteasome system (UPS) (Hershko A, Ciechanover, 1998;

Willems *et al.*, 2004; reviewed by Ravid and Hochstrasser, 2008). Here a poly-ubiquitin chain is added to a substrate, and presented to the 26S proteasome, the protein complex responsible for the cleavage of the tagged proteins into short peptides. By degrading short-lived regulatory or structurally aberrant proteins, the UPS controls many cellular processes, including the cell cycle, signal transduction, programmed cell death, immune responses, metabolism, protein quality control and development (Varshavsky, 2005; Tanaka, 2009). It has now become clear that also mono ubiquitination has an important role, mainly in protein localization and activity; this will be briefly discussed.

## 2.1 Structure of UPS

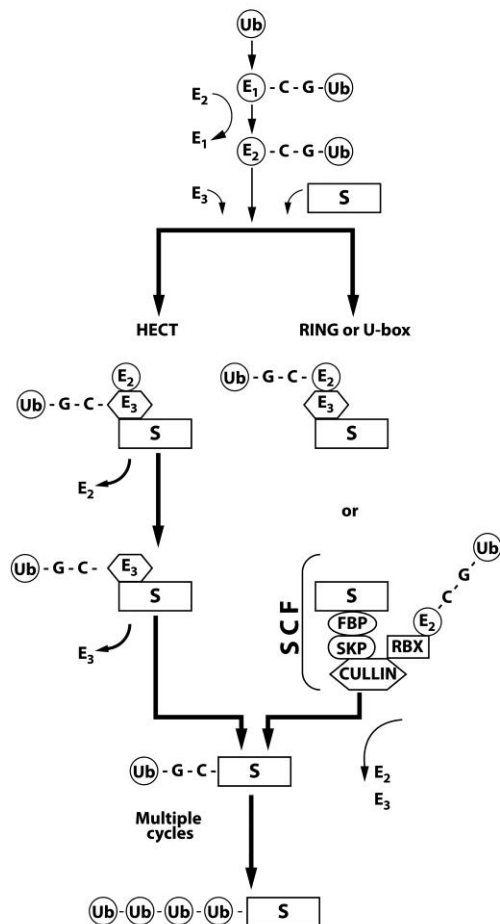
E3 Ubiquitin (Ub) ligases mediate proteolysis by covalent attachment of Ub molecules (poly-ubiquitination) to specific residues of target proteins (Hershko and Ciechanover, 1998). Ub is a highly conserved protein of 76 aminoacids present in all eukaryotic cells (initially known as ATP-dependent proteolysis factor 1, APF-1, (Hershko and Ciechanover, 1980) that is attached to proteins by the sequential action of E1-, E2- and E3-Ub ligase proteins (Pickart, 2001; Wilkinson, 2000). An Ub activating enzyme (E1) and a Ub-conjugating enzyme (E2) are responsible for the activation and conjugation of Ub molecules that are finally linked to selected target proteins by an E3-Ub ligase (Figure 2). To date, two main types of E3-Ub ligases have been described: those that bind Ub molecules donated by E2 before transferring the Ub moieties to the target protein, known as HECT (homologous to E6-AP C-terminus) E3, and those that directly catalyze the Ub transfer from E2-conjugating enzyme to the E3-bound target protein, the RING (Really Interesting New Gene) domain and the U-box domain-containing proteins (reviewed by Dreher and Callis, 2007). In some cases, polyubiquitination requires the additional activity of certain ubiquitin-chain elongation factors denominated E4 enzymes (Koegl *et al.*, 1999; Kuhlbrodt, Mouysset and Hoppe, 2005).

For formation of the polyubiquitin chain a linkage between C-terminal Gly<sup>76</sup> of one Ub moiety and the internal Lys<sup>48</sup> of the previously conjugated moiety is required. Only Lys<sup>48</sup>-based Ub chains are recognized by the 26S proteasome and serve as proteolytic signals. Findings indicate that for several proteins, the first Ub moiety can also fuse linearly to the alpha-NH<sub>2</sub> group of the N-terminal residue (N-end rule). However, the next Ub molecule joins via Lys<sup>48</sup> to the first linearly fused Ub. N-terminal ubiquitination is then considered as a targeting option for degradation of proteins that naturally lack lysine (Ciechanover *et al.*, 2004).

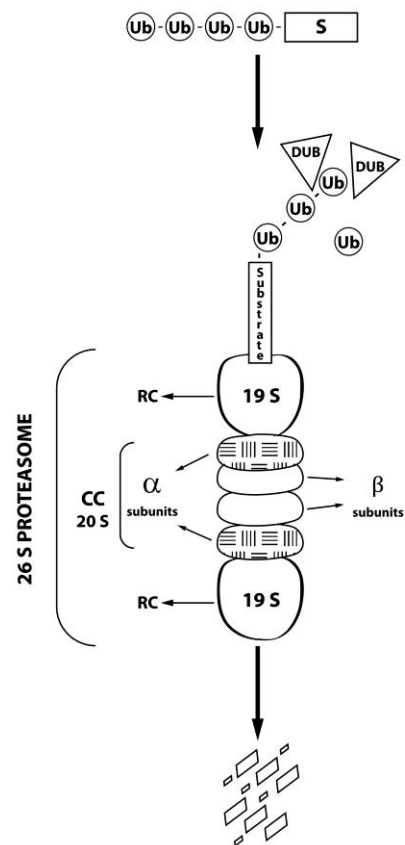
Ub-E3 ligases present the earmarked substrate to the proteasome, a large proteinase complex with multiple catalytic domains that can be located in the cytosol or in the cell nucleus (Peters, Franke and Kleinschmidt, 1994). The apparent sedimentation coefficient of the active proteasome is 26S, reason why the complex is usually referred to as the 26S proteasome. It consists of a multi-catalytic protease complex of 2.5 MDa having a 20S core protein (CP) complex, and one or two S19 regulatory particles (RP), or caps. Four stacked rings, two identical outer  $\alpha$  rings and two identical inner  $\beta$  rings, form the 20S catalytic subunit. The catalytic sites are localized to some of the  $\beta$  subunits showing six unique ATPdependent serine protease sites. The 19S RP is composed of 17 distinct subunits that

serve to recognize ubiquitinated proteins, probably playing also a role in their unfolding and translocation into the interior of the 20S CP (Tanaka, 2009).

A.



B.



**Figure 2. Structure of ubiquitin proteasome system.** Scheme representing the steps involved in ubiquitination of a protein substrate via HECT or RING/U-box type E3-ubiquitin ligases **(A)** and its degradation by the 26S proteasome **(B)**. The SCF complex is a Cullin RING E3-Ligase (CLRs) which consists of a CULLIN as a core scaffold protein, and several adaptors such as SKP1, RBX, and a F-box protein (FBP) responsible of substrate recognition.

Ubiquitin (Ub) binds initially to E1 for its activation, then to E2 for its conjugation and finally to an E3 ligase. Transfer of the Ub molecule to a protein recognized by E3 leads to an earmarked polyubiquitinated substrate with Ub moieties covalently linked together via their lysine residue (K48), which is recognized for proteasome-dependent degradation. After binding to the regulatory cap (RC) of the 26S proteasome, deubiquitinating enzymes (DUB) assist with the release of Ub monomers from the substrate, which is further degraded inside the proteasome catalytic core (CC) resulting in the production of small peptide products.

E1: Ub activating enzyme; E2: Ub conjugating enzyme; E3: Ub ligase; C: cysteine; G: glycine; HECT: homologous to E6-AP C-terminus; RING (Really Interesting New Gene); SKP (S-phase kinase associated protein/suppressor of kinetochore protein); FBP (F-box protein); RBX (RING-H2 motif containing protein); S: substrate.

The Anaphase-promoting complex (APC) and the Skp1-Cullin-F-box protein complex (SCF complex) are two examples of multi-subunit E3s involved in recognition and ubiquitination of specific target proteins for degradation by the proteasome. Among the RING-E3 Ub ligases, those forming the SCF complex are particularly prevalent and

important. SCF complexes, are made up of a core of three main proteins: Cullin (CUL1), RING-H2 motif containing protein (RBX1), also known as HRT1 or ROC1 (Tyers and Willems, 1999), and the S-phase kinase associated protein/suppressor of kinetochore protein (SKP1), which recruits a fourth subunit called the F-box protein (FBP) (Dharmasiri and Estelle, 2002). The presence of the FBP determines the specificity of the SCF complex, as the FBP recruits the proteins that are targeted for degradation (Patton *et al.*, 1998; Tyers and Willems, 1999).

## **2.2 Function of the UPS in plants**

In *Arabidopsis* several genes coding for UPS members have been identified. So far, only two genes coding for Ub-E1 activating enzyme (Hartfield *et al.*, 1997) and 37 genes for Ub-E2 conjugating enzyme, have been reported (Kraft *et al.*, 2005). However, many more genes encoding Ub-E3 ligases, including seven HECT type, 61 U-box domain and hundreds of RING domain containing proteins are present (reviewed by Dreher and Callis 2007). The core protein of the RING type E3 ligase belongs to the cullin family, reason why members of this group are denominated Cullin RING ligases (CRLs). Based on identity with vertebrate cullins, 11 CUL-like proteins have been identified in *Arabidopsis* (Shen *et al.*, 2002), six of which have been implicated in CRLs. Co-precipitation of CUL1 with HA-tagged ASK1, one of 21 predicted *Arabidopsis* SKP family members (called ASKs for *Arabidopsis* SKP1-like) has been observed in *in vitro* experimentation (Farras *et al.*, 2001). F-box proteins (FBPs) contain a domain originally found in a SKP/ASK interaction motif of mammalian cyclin F (Bai *et al.*, 1996) that bind simultaneously to a SKP/ASK protein and to an UPS substrate. Sequence analysis in *Arabidopsis* have revealed more than 700 F-box proteins that may interact via their F-box with ASK members (Gray *et al.*, 1999; Gagne *et al.*, 2002; Kuroda *et al.*, 2002; Risseuw *et al.*, 2003; Takahashi *et al.*, 2004). The UPS plays an essential role in almost all processes of growth and development and adaptation in plants. It has been found that F-box proteins play an important role for instance in phytohormone perception and signalling, in control of the circadian rhythm and in photomorphogenesis and flower development (Gray *et al.*, 1999; Samach *et al.*, 1999; Dieterle *et al.*, 2001; Xu *et al.*, 2002). Below I shall summarize some more details about the essential role of the UPS in plant growth factor perception and signaling.

The plant growth regulator auxin plays a role in almost all developmental processes. Auxin responsive genes are controlled by transcription factors denominated auxin response factors (ARFs). The cognate auxin/indole-3-acetic acids (Aux/IAA) proteins interact with these ARFs thus blocking their transcriptional activity. Degradation of Aux/IAA proteins allows then the expression of auxin response genes. Recently, it was shown that the F-box protein TIR1, which recognizes Aux/IAA as targets for degradation, has the property to directly bind auxin. This showed for the first time the ability of an ubiquitin ligase to act as a hormone receptor. This process of hormone perception by TIR1 is assisted by a small cofactor denominated inositol hexakisphosphate (IP6) that binds close to the auxin-binding site in TIR1. After binding of auxin and IP6 TIR1 can bind more efficiently to Aux/IAA

proteins, accelerating their ubiquitination and degradation (Tan *et al.*, 2007; Tan and Zheng, 2009).

Similarly, the F-box protein COI1 has been reported to bind a different plant hormone, jasmonic acid (JA), and induce the targeting for degradation of the jasmonate ZIM domain (JAZ) proteins. These JAZ proteins act as co-repressors of JA induced genes (reviewed by Somers and Fujiwara, 2009). Gibberellins (GA) promote seed germination, elongation growth and flowering; the F-box proteins (FBPs) SLEEPY 1 and SNEEZY 1 in *Arabidopsis*, and GID2 in rice, mediate the proteolysis of the DELLA proteins, repressors of the response to gibberellins. However, in this case FBPs do not act as receptors of GA; instead a protein denominated GA insensitive dwarf (GID1) is the receptor; this protein, interacts with the FBPs and stimulates DELLA protein turnover (reviewed by Vierstra, 2009).

The UPS also controls the processes of plant development and stress response that are regulated by ethylene and abscisic acid (ABA). The ethylene transcriptional response is triggered by the ethylene insensitive 3 (EIN3) transcription factor, and probably, by five additional EIN3-like proteins (EIL1-5). The EIN3 transcription factor is targeted by the FBPs EIN3 binding box protein 1 (EBF1) and EIN3 binding box protein 2 (EBF2) for proteolysis. Remarkably, contrary to the three phytohormones described above, ethylene induces the accumulation of EIN3 instead of promoting its FBP-mediated degradation. Although the mechanism of how this happens is not yet clear, it is believed that ethylene may induce the modification of EIN3 and EIL1 in a way that prevents their recognition by SCF<sup>EBF1/EBF2</sup>, allowing their accumulation to a level high enough to direct ethylene dependent transcription and protein synthesis (reviewed by Vierstra, 2009; Ho *et al.*, 2008). Recent studies by An *et al.*, (2010) demonstrate how ethylene regulates EIN3 and EIL1 and clarify that EIN2 is required for ethylene-induced EIN3/EIL1 stabilization, and not MKK9, a kinase previously reported to directly phosphorylate EIN3 and modulate its stability (Yoo *et al.*, 2008). Their analysis also reveals that EIN3/EIL1 accumulate due to the proteosomal degradation of the F-box proteins EBF1 and EBF2 in the presence of ethylene.

The plant growth regulator ABA plays an important part in plant responses to environmental stress and pathogens (Zhu, 2002; Seo and Koshiba 2002). In response to drought ABA is produced in plant roots and then translocated to the leaves, where it induces stomatal closure preventing further water loss from the leaves in times of low water availability. The ABA responsive transcription factors ABA-insensitive 3 (ABI3) and ABA-insensitive 5 (ABI5) are degraded via the UPS; whereas degradation of ABI3 is enhanced by ABA after stimulation of the synthesis of the E3-ligase ABA-interacting protein 2 (AIP2), the degradation of ABI5 is blocked, probably by preventing its interaction with the keep on going (KEG) E3 ligase responsible for ABI5 protein turnover. Downstream of ABI3 and ABI5, the DRE (dehydration-responsive element)-binding protein 2 (DREB2) family of transcription factors, promotes the expression of stress-protective proteins. One member so far, the DRE2A protein has been found to be degraded via the E3 ligases DRE2A-interacting protein DRIP1 and DRIP2. Apparently, turnover of this protein is required for a proper response to abiotic stress conditions (reviewed by Vierstra, 2009). Finally, the FBP called more axillary growth 2 (MAX2) was found recently to be required for the perception of the newly



discovered plant hormone strigolactone, which controls axillary shoot development (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008).

### **2.3 UPS and defense response**

Pathogen infection, wounding, and presence of the plant growth regulators ethylene JA and SA, which play an important role in the defense response of the plant, leads to up-regulation of the ubiquitin-activating enzyme E1 in tobacco (Takizawa *et al.*, 2005). Similarly, specific E3 ligase components including CRL, non-CRL and U-box domain proteins may be induced in a response to infection by general elicitors and avirulence factors (reviewed in Zeng *et al.*, 2006). The regulated proteolysis of endogenous or pathogen produced proteins can contribute to either basal, local R-mediated/Hypersensitive Response (HR), systemic acquired resistance (SAR) or induced systemic (ISR) resistance. Also ethylene, JA and SA, plant growth regulators that employ the UPS for signal perception and/or transduction, induce defense responses and the expression of defense genes. Pathogens trigger such defense responses by the release of pathogen associated molecular patterns (PAMPs) or microbial associated molecular patterns (MAMPs), such as lipopolysaccharides (LPS), peptidoglycans, flagellin, microbial cell wall fragments, phospholipids, proteins, double stranded RNA and methylated DNA. These elicitors are recognized by so called host pattern recognition receptors (PRRs), which are often present on the cell surface (reviewed by Iriti and Faoro, 2007). PRRs include the Toll-like receptors (TLRs) in plants and animals, flagellin sensitive 2 (FLS2), a leucine-rich repeat receptor kinase (LRR-RK) and the previously mentioned EF-Tu receptor EFR in plants. This leads to the establishment of innate immunity, which protects the plant (animals) against non-pathogenic invaders. The innate immune system of animals and plants is a first line of defense against pathogens. Pathogenic micro-organisms have evolved in parallel with their eukaryotic hosts, and developed strategies to counteract any kind of innate immune responses that would interfere with their pathogenesis. During this co-evolution, both plants and animals have in response increased their capacity to recognize microbial elicitors of different nature. Elicitation of the receptor leads to activation of the MAPK pathway which triggers in turn the transcription of genes involved in defense (Nakagami *et al.*, 2005).

The responses to flagellin and other PAMPs have been called PAMP-triggered-immunity (PTI). Some pathogens however, have evolved ways that enable them to escape PTI by secretion of specific effectors into the host cells. These virulence factors are encoded by the avirulence (*avr*) genes of these pathogens. This has led plants to develop a second layer of defense called effector-triggered immunity (ETI), by which plants can perceive such effectors through additional receptors, encoded by plant resistance genes (R), typically nucleotide-binding leucine-rich repeat (NB-LRR) that ensure the perception of pathogen race/strain specific Avr proteins. R proteins contain conserved structural domains including N-terminal coiled coil (CC), or toll-interleukin 1 receptor (TIR)-like domains, central nucleotide binding site (NBS), and C-terminal leucine rich repeat (LRR) domains (Martin *et al.*, 2003). Some of these proteins do directly interact with the Avr proteins to perceive the presence of the pathogen, but others also recognize them indirectly, acting as guards of other hosts

proteins targeted by the pathogen effectors (reviewed by Selote and Kachroo, 2010). Such R-mediated resistance is race-specific (reviewed by Boller and He, 2009; Iriti and Faoro, 2007).

### 2.3.1. UPS and plant pathogen effector proteins

Several pathogenic bacteria translocate virulence proteins or effectors to their animal or plant hosts via the T3SS and the T4SS. Once inside the host, these effectors play a specific role in favor of the pathogen. In animal pathogens some of these proteins such as SopA, SopB, SopE and SptP (from *Salmonella* spp); YopE, YopE (from *Yersinia* spp) and ExoU (from *Pseudomonas aeruginosa*), use the host UPS to regulate their degradation rate in order to adjust their concentration properly according to the bacterial needs. Others have acquired the capacity to interfere with the ubiquitination level of key cell proteins of the innate immune signaling cascade, thus preventing a strong host defense response. These kinds of effector proteins are secreted by both animal pathogens such as *S. typhimurium*, *Y. pseudotuberculosis* and *Y. pestis*, and plant pathogens such as *Xanthomonas campestris*, which causes black rot (Hotson and Mudgett, 2004, Zhou *et al.*, 2005). Translocated effectors such as YopJ of *Y. enterocolitica* have acetyltransferase activity and modify critical residues in the activation loop of MAPKK thereby preventing phosphorylation and thus interfering with the MAPK signaling pathways and the defense response (Mukherjee *et al.*, 2006). The effector protein OspG (*Shigella flexneri*) is able to interact with ubiquitinated E2 Ub-conjugating enzymes and negatively regulate the NF- $\kappa$ B inflammatory response by interfering with the proteasome-dependent degradation of inhibitor of NF- $\kappa$ B (I $\kappa$ B $\alpha$ ) (reviewed by Angot *et al.*, 2007).

### 2.3.2. Pathogen effectors are able to mimic plant F-box proteins

Certain pathogens have evolved more advanced ways to interact with their host by mimicry of E3 ligase subunits, thereby manipulating the host UPS to their advantage. The bacterium *Ralstonia solanacearum* translocates effector proteins with an F-box into their host, using the T3SS. These proteins possess both a leucine rich repeat (LRR) and an N-terminal F-box domain by which they interact with several ASK members. Due to the characteristic aminoacid sequence located at their LRR, they are known as GALA proteins. Deletion analysis of several GALA proteins can affect the virulence of the bacteria in *Arabidopsis* and tomato; however, research on *Medicago truncatula* showed that specific deletion of the F-box domain of the GALA7 protein can severely affect the virulence of this pathogen (Cunnac *et al.*, 2004; Angot *et al.*, 2006). This suggests that similar to the *A. tumefaciens* VirF protein, single or associated GALA proteins could form part of SCF E3-Ub ligases in eukaryotic host cells responsible for the ubiquitination of targets involved in plant defense. The VirF protein is translocated to plant cells via the T4SS of *A. tumefaciens*, where it plays a relevant function in host range determination and virulence on certain specific host plants (Hooykaas *et al.*, 1984; Melchers *et al.*, 1990). VirF carries an F-box domain by which it interacts with ASK proteins, members of the SCF E3-Ub ligase (Schrammeijer *et al.*, 2001). It is predicted that during the infection process VirF may be responsible for the recognition of

substrates targeted for degradation inside the host plant. Therefore, in the present work we mainly focused on the search for the possible target proteins recognized by VirF within host cells.

Attempts to demonstrate similar properties of an additional F-box containing protein belonging to *Mesorhizobium loti* (Msi061) showed that this protein was translocated into plant cells via the *A. tumefaciens* T4SS (Hubber *et al.*, 2004). Additional *in silico* analysis of other organisms for which the genomic sequence was determined revealed the presence of F-box containing proteins and Ub-E3 related subunits in other bacteria that interact with eukaryotic organisms, suggesting that a wide range of bacteria may contain putative FBPs (*Legionella pneumophila*, Cazalet *et al.*, 2004; *Coxiella burnetii*, Seshadri *et al.*, 2003; *Candidatus Protochlamydia amoebophila* UWE25, Horn *et al.*, 2004). Further analysis is required to determine whether these proteins are indeed translocated to host cells via the T3SS or T4SS and to unravel their specific role in connection to the UPS and infection (Angot *et al.*, 2007).

## **2.4 Ub and Ub-like related processes not associated to protein degradation but protein regulation**

Proteins containing ubiquitin-binding domains (UBDs) interact with ubiquitinated targets and regulate diverse biological processes. Many of the UBD-containing proteins are also themselves monoubiquitinated. The activity of Ub-receptors is then controlled by coupled monoubiquitination, which provides an efficient switch from an active to an inactive conformation (Hoeller *et al.*, 2006, Hoeller and Dikic, 2010).

During the last decade, studies have demonstrated that the attachment of Ub to proteins does not always lead to their degradation, but may lead to their activation or re-routing. A number of additional peptides have been found to be conjugated to proteins in a similar way as Ub, reason why these are known as ubiquitin-like proteins (UBLs). Addition of these small proteins may determine sub-cellular destination or protect proteins from ubiquitination and in this way, be involved in the regulation of several cellular processes including cell cycle and division, growth and differentiation, transcription, apoptosis, immune and inflammatory response, signal transduction, receptor mediated endocytosis, metabolic pathways, and cell quality control (reviewed by Ciechanover, 2006).

Ub-like molecules can be classified into two groups: Ub-domain proteins (UDPs) and Ub-like protein modifiers (ULMs) (Kerscher *et al.*, 2006; Walters *et al.*, 2004; Welchman, Gordon, Mayer, 2005). UDPs carry a sequence that is similar to Ub, but they are not conjugated to proteins. Instead, they fulfil adaptor function, binding noncovalently to Ub or ULMs and its associates via an Ub-interaction motif or Ub-associated (UBA) domain. An example of such proteins is the Rpn10 subunit of the 19S proteasome, which assists in the direct recognition of polyubiquitinated proteins by the 26S proteasome. The ULMs on the other hand, are conjugated to proteins and function in an Ub-like manner. By attachment of ULMs, a conformational change is created in a protein that may alter the binding capacity of the protein to another molecule, with direct consequences that may affect its function, localization and/or half-life. Among these proteins we find the neural precursor cell-expressed developmentally downregulated-8 (NEDD8, Rub1), which plays a role as a regulator of Ub-

E3 ligases (cullin). Other known members of this group are the small Ub-related modifiers (SUMO-1, 2 and 3; Saitoh and Hinchey, 2000), which appear to have various functions in the cell (Hay, 2005). With more than 100 proteins identified as substrates for the SUMO system, it seems that sumoylation is involved in many cellular processes including nucleocytoplasmic transport, protein-protein interactions, protein-DNA-binding activity, protein stability, and in genome organization, repair and transcription. It is worth to mention that at least eight more ULMs exist in mammals, which include interferon-stimulated gene-15 (ISG15), Finkel-Biskis-Reilly murine sarcoma virus (FBRMuSV)-associated ubiquitously expressed gene (Fau) Ub-like protein (FUB1), F-adjacent transcript-10 (FAT10), autophagy-defective 8 and 12 (APG8, APG12), Ub-related modifier-1 (URM1), Ub-like protein-5 (UBL5) and Ub-fold modifier-1 (UFM1) (reviewed by Herrmann, Lerman and Lerman, 2007).

### 3. AIM OF THE WORK

The *Agrobacterium* virulence system resembles in many aspects the conjugative systems determined by certain broad host range plasmids (Pansegrau and Lanka, 1996). This unique capacity to mediate DNA transfer to plants is mediated by accessory virulence (*vir*) genes. One of these, *virF*, found in octopine strains, encodes a hydrophilic protein that plays a role as a host range determinant (Melchers *et al.*, 1990). Its presence apparently increases the efficiency of the transformation process (Regensburg-Tuink and Hooykaas, 1993). The Cre Reporter Assay for Translocation (CRAFT) assay was developed to directly and efficiently detect translocation of *Agrobacterium* effector proteins such as VirE2, VirF and VirE3 into host cells (Vergunst *et al.*, 2000; Schrammeijer *et al.*, 2003). The first phase of this thesis aimed to study the transport of several Vir proteins into the plant cell such as VirE1, VirE3 and VirD2 using the CRAFT assay in both *Arabidopsis* and yeast.

VirF carries an F-box domain by which it interacts with the plant homologues of the yeast Skp1 protein, ASK1 and ASK2 (Schammeijer *et al.*, 2001). Skp1 forms part of the SCF E3-Ub ligase complexes with Cdc53 (cullin), Cdc34 (an Ub-conjugating enzyme) and various F-box proteins. The F-box proteins in these complexes function as receptors that recruit substrates for destruction. The present project aimed to identify VirF interacting host proteins, and study the interaction between VirF and its eukaryotic binding partners in more detail in models such as *Arabidopsis* and yeast.

### 4. OUTLINE OF THE THESIS

In a time with a shortage of food supplies for the still increasing human population, my main concern when I started this project was how basic research on *Agrobacterium* could contribute to reduce the big gap existing between modern biotechnology and real agricultural practices, and in how this could be applied to the improvement of the quality of life of small and medium scale farmers in the developing world. Modern biotechnology offers new opportunities to increase the competitiveness and sustainability of agriculture. Transgenic resistant varieties are now rapidly moving towards commercialization and studies directed to

the cloning of resistance genes, and to the understanding of plant interactions with fungal and bacterial pathogens, have demonstrated agro-economically useful levels of plant disease resistance. The study of *Agrobacterium* as a model system has opened the comprehension of how pathogens deliver macromolecules into target cells, making possible the improvement of transformation techniques towards important crop species.

In **Chapter 2** we analyzed whether the *A. tumefaciens* proteins VirD2, VirE1 and VirE3 were translocated to plant cells by the T4SS in absence of T-DNA by a similar mechanism as VirE2 and VirF. The experiments revealed that VirD2 and VirE3 are directly translocated from *A. tumefaciens* into host cells via the VirB/VirD4 transport pore.

The *virF* gene encodes a protein that harbors a F-box domain at the N-terminus, known to interact with the plant homologues of the yeast SKP1 protein, the *Arabidopsis* SKP1 proteins (ASK1 and ASK2) and to be translocated to host cells via the T4SS. As part of the SCF complexes, F-box proteins (FBPs) are known to be the responsible for the recognition of proteins designated for degradation by the 26S proteasome. To identify such putative targets in **Chapter 3** we used a VirF protein devoid of the F-box domain as bait in a yeast two-hybrid analysis. Here we identified a set of diverse host proteins from which five were confirmed by GST-pull down assays to be direct interaction partners (PIF proteins). To better characterize the regions of VirF involved in target recognition, in **Chapter 4** deletions of VirF were used in GST-pull down assays with four interacting proteins; it was found that two different regions may be involved in protein recognition but that this could depend on the nature of the target protein.

The precise function of VirF *in planta* is not clear; therefore, to confirm whether VirF has any activity in recognizing a specific target and mediate its degradation, in **Chapter 5** we used an *in vivo* approach by co-expressing in yeast cells VirF with the VirE2 interacting protein (VIP1), a previously suggested interactor, as well as with some of the putative interactors of VirF or PIF proteins. We found preliminary evidence for the degradation of VIP1 and one of the putative interactors of VirF (PIF). In addition, in **Chapter 6** we analyzed whether VirF interacts with ASK1 and other subunits of the SCF complexes *in vivo* by using co-immunoprecipitation analysis after expression of a tagged version of VirF in *Arabidopsis* cell suspensions. The results showed that VirF was localized both in the cytoplasm and also in the nucleus; also it was found that VirF co-precipitated with the plant SCF members ASK1 and CUL1. **Chapter 7** comprises the general summary and discussion.

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