# Multiple faces of Fusarium oxysporum effector protein Avr2

Di, X.

# Chapter 6

## General discussion

The soil-inhabiting fungus *Fusarium oxysporum* (*Fo*) causes vascular wilt disease on a wide range of plants provoking severe economically losses. Though *Fo* species have been reported to collectively infect more than 120 different hosts, each *forma specialis* (f.sp.) is specific for one or a very limited number of host species only (Michielse and Rep, 2009). Over last decades the interaction between *Fo* f.sp. lycopersici (*Fol*) and tomato developed into a prime model to study the molecular mechanisms underlying disease and resistance to wilt causing pathogens (Takken and Rep, 2010). At the start of this project it had been reported that *Fol* effector protein Avr2, originally identified in the xylem sap of *Fol* infected tomato plants, is an important pathogenicity factor (Houterman et al., 2009). Furthermore, Avr2 had been shown to be recognized inside the plant nucleus by the tomato resistance protein I-2 (Ma et al., 2013) (Figure 1A). *Fol* races that overcome I-2-mediated resistance have been found to either carry specific point mutations or to contain a small deletion in *Avr2*. The point mutations result in single amino acid changes in the protein and the deletion in the loss of a single amino acid. None of these mutations compromise the virulence function of Avr2, but the protein is no longer perceived by I-2 (Houterman et al., 2009; Chellappan et al., 2016). In this chapter, current insights in Avr2 function and its possible mode of action are discussed.

**Does Avr2 manipulate hormone signaling in tomato upon *Fol* infection?**

The involvement of major phytohormones, such as jasmonic acid (JA), ethylene (ET) and salicylic acid (SA), in the susceptibility of tomato against *Fol* has been studied and the results are described in Chapter 3. Tomato mutants affected in either the production- or perception of one of the above-mentioned hormones were inoculated with either a wild-type *Fol* strain 007 (*Fol007*) or the less virulent strain *FolΔAvr2* in which the *Avr2* gene has been knocked out. Tomato plants in which SA is degraded due to the expression the salicylate hydroxylase transgene (*NahG*), were found to be more susceptible to infection by both *Fol007* and *FolΔAvr2* than wild-type tomato plants. Together with the observation that Avr2 suppresses pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) responses (Chapter 5), this suggests that *FolΔAvr2* causes less disease symptom in susceptible tomato plants due to its inability to suppress PTI. SA plays an important role in PTI signaling (Tsuda et al., 2009) and indeed pathogenicity of *FolΔAvr2* strains was restored on *NahG* plants. Actually the plants became even more diseased than wild-type tomato plants, suggesting that the role of SA in restricting *Fo* is not limited to its involvement in PTI.

In contrast to the hyper-susceptibility of *NahG* plants, tomato lines compromised in ET production due to the constitutive expression of the bacterial 1-amino-cyclopropane-1-carboxylic acid deaminase gene (*ACD*), exhibited less disease symptoms than wild-type tomato plants upon *Fol* infection. In addition, also the *Never ripe* (*Nr*) mutant impaired

in ET perception, showed a reduced susceptibility, indicating that both ethylene production and perception is required for disease symptom development. It has been shown that ETHYLENE INSENSITIVE3 (EIN3) and ETHYLENE INSENSITIVE3-LIKE1 (EIL1), known to mediate ET signaling, negatively regulate PTI resistance (Chen et al., 2009). Combined with our observation, it is possible that the ET signaling pathway positively contributes to the suppression of the PTI response by Avr2.

Compared to wild-type plants colonization of the vasculature by *Fol007* was reduced in trangenic plants in which expression of the *prosystemin* gene is driven by the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter. Prosystemin is a positive regulator of JA signaling and these plants constitutively accumulate high levels of proteinase inhibitor proteins, thereby mimicking a constitutive JA signaling phenotype (Howe and Ryan, 1999). This implies that JA signaling restricts the colonization potential of the pathogen. Therefore it was interesting to observe that, whereas colonization was decreased for the wild-type fungus, the *FolΔAvr2* strain showed increased colonization of *35S:prosystemin* plants. This indicates that Avr2 might induce the JA signaling pathway, which unintentionally restricts infection. Effectors other than Avr2 might also manipulate JA signaling. Gawehns and coworkers have proposed a model in which SIX8 interferes with JA signaling via its interaction with the transcriptional regulator TPL(TOPLESS)/TPR(TOPLESS-related). TPL/TPR was shown to interact directly with JAZ proteins that are involved in JA signaling (Gawehns, 2014). Accordingly, it is plausible that Avr2 triggers JA signaling reducing host colonization.



**Where does Avr2 exert its virulence function?**

Plant pathogenic microbes secrete effector proteins to suppress or evade plant immune responses. The strategies used by various pathogens to deliver their effector proteins differ. Many pathogenic bacteria secrete their effectors directly into the plant cells by a type III secretion system. Pathogenic fungi and oomycetes, do not inject their effectors inside the cell and they secrete them into the extracellular spaces. Some pathogens like the fungus *Cladosporium fulvum*, secrete their effectors into the apoplast. Others, like the oomycete *Phytophthora infestans*, form feeding structures that invade the plant cell, but not breach the plasma membrane, and here the effectors are secreted in the extracellular spaces between plant- and fungal plasma membrane. (Dodds and Rathjen, 2010; Stergiopoulos and de Wit, 2016). In the tomato-*Fol* pathosystem, Avr2 has been isolated from the xylem sap of *Fol* infected tomato plants (Houterman et al., 2007). As Avr2 exerts both its virulence and avirulence activity intracellularly, translocation of the protein into tomato cells is suggested (Ma et al., 2013) (Chapter 4). In addition, in transgenic *Avr2* tomato in which Avr2 is secreted into the apoplast, the virulence defect of a *FolΔAvr2* strain is fully complemented, again implying that Avr2 is taken up

by host cells. Yet, grafting experiments and crosses of *I-2* plants with transgenic *Avr2* tomato, revealed that cells do not spontaneously internalize Avr2 from the apoplast or the xylem sap, as I-2-mediated immune signaling was not triggered. This apparent paradox was resolved when we observed that infiltration of *Agrobacterium tumefaciens* in leaves of *Avr2/I-2* plants triggered *I-2*-mediated cell death, which indicates that the presence of a pathogen is required to trigger effector uptake. In the *Avr2/I-2* plants Avr2 expression was driven by the strong constitutive 35S promoter (Kay et al., 1987). Instead of triggering re-uptake of secreted protein, it cannot be excluded that the presence of *Agrobacterium* interferes with Avr2 secretion in the transgenic plants. But if so, this would result in the formation of a cytosolic pool of Avr2 that triggers I-2 activation. To confirm uptake, agro-infiltrations could be done on *I-2* scions grafted on an *Avr2* rootstock. If *I-2*-mediated cell death occurs following infiltration, this would confirm that *Agrobacterium* does facilitate Avr2 uptake from the xylem sap into cells.

As *Fol* and *Agrobacterium* can both facilitate Avr2 accumulation in the cytosol, there must be a shared property between these organisms that triggers effector uptake by the plant. The observation that *V. dahliae* failed to trigger Avr2 uptake suggests that it either does not contain this property or that the amount of uptake was not sufficient to be monitored in our experimental setup. The amount of fungal biomass produced by the fungus during infection might have been too low to trigger detectable uptake (Faino et al., 2012) (Chapter 4). To explore the possibility that *V. dahliae* is capable of inducing effector uptake, the *Avr2/I-2* plants could be inoculated with *V. dahliae*. If upon infection the plants mount an I-2-mediated deference response prohibiting further colonization, this would provide support for the hypothesis that *V. dahliae* does facilitate Avr2 uptake and that this property is not confined to *Fol* and *A. tumefaciens*.

Proteins may enter plant cells via a) endocytic uptake or b) a transmembrane transporter (Drin et al., 2003; Goldberg and Cowman, 2010). Endocytosis, the vesicular uptake of extracellular macromolecules, is the main mechanism for internalization into cells. Endocytosis pathways can be subdivided into five categories: clathrin-mediated endocytosis, receptor-mediated endocytosis, caveolae-mediated endocytosis, lipid raft-mediated macropinocytosis and phagocytosis (Conner and Schmid, 2003; Khalil et al., 2006; Mulcahy et al., 2014). After endocytosis, the internalized molecules are present in endosomes, separated by a membrane from the host cytosol. Endosomes can either fuse with lysosomes for degradation of their content or they recycle their content back to the cell surface. Alternatively, internalized cargo can be released into the cytoplasm via retrotranslocation through intracellular vesicles such as the Golgi apparatus and endoplasmic reticulum (ER) (Perotto and Baluška, 2012). Retrotranslocation is a process in which mis-folded proteins are transported from the ER into the cytosol, where they are targeted for ubiquitin/proteasome-mediated degradation (Ellgaard and

Helenius, 2003). So proteins exiting via the retrotranslocon route are typically directly degraded upon their entrance of the cytosol. Therefore, it is very unlikely that effector proteins enter plant cells via endocytosis since it would require a way to escape from proteasomal degradation (Khalil et al., 2006).

Recent data revealed that some effectors utilize specific motifs to interact with the plasma membrane, presumably to facilitate their uptake. Effector AvrM of the flax rust pathogen *Melampsora lini* for instance, has been shown to translocate into host cells via an N terminal domain that mediates binding to the plant plasma membrane (Ve et al., 2013). AvrM binds phosphatidylinositol-3-phosphate (PI3P). However, studies with AvrM deletion mutants showed that PI3P binding activity is not necessary for protein uptake (Gan et al., 2010). The RxLR (Arg-x-Leu-Arg) motif in oomycete effectors also binds PI3P (Kale et al., 2010). PI3P is proposedly present on the outer surface of the plant plasma membranes facilitating translocation of the interacting protein into host cells (Whisson et al., 2007; Dou et al., 2008). Kale and co-workers proposed that an RxLR-like motif is present in Avr2, which is required for PI3P binding and protein uptake (Kale et al., 2010). However, mutating the RxLR-like motif, represented by the "RIYER" sequence in Avr2, resulted in a protein that could no longer trigger I-2-dependent cell death, even when expressed without its signal peptide to ensure a cytosolic location (Ma, 2012). Furthermore, based on the structure of Avr2 (Chapter 5), the "RIYER" sequence is part of a structural motif and partially buried in the structure, making it unlikely that it is available for an interaction and involved in the uptake process. So currently it is unclear whether PI3P binding is required for Avr2 uptake or whether other mechanisms are involved.



Whereas the N terminus of AvrM is necessary and sufficient for its internalization into plant cells (Rafiqi et al., 2010), its C-terminal region is required for AvrM-dependent Effector Triggered Immunity (ETI) (Catanzariti et al., 2010). AVR3a is an effector of the oomycete *Phytophthora infestans*. Like AvrM, the N-terminal region of AVR3a, which includes the signal peptide and RXLR motif, is required for uptake, while the C-terminal region encodes the effector domain required for activation of R3a-dependent immunity and suppression of INF1-induced cell death (Bos et al., 2006). These observations suggest that effectors can have two distinguishable domains, one for translocation and one for (a)virulence function. Avr2 does not have two clearly distinguishable domains, but it has been shown that the extreme N-terminal region (Δ37 truncation) of Avr2 is not required for I-2-mediated cell death, when the protein is expressed without signal peptide (Ma et al., 2013). Therefore, it is plausible that the extreme N-terminal region is involved in protein uptake. To examine this possibility, one could express the Δ37 Avr2 variant, carrying a signal peptide for secretion, in a *Fol*Δ*Avr2* strain and assess its ability to complement (a)virulence. Alternatively, one could co-express it with *I-2* in

*Nicotiana benthamiana* and monitor whether it triggers I-2-mediated cell death. Also, the Δ37 region might be fused to a reporter such as the green fluorescent protein to monitor its uptake in plant cell. As positive control full length Avr2 fused to the same tag could be used. As has been suggested in Chapter 4, that the fluorescent tag most likely will be cleaved off in the tomato apoplast, impeding the interpretation of the data, these assays should be done in *Arabidopsis thaliana* in which cleavage is not expected to occur (van Esse et al., 2006).

**Can pathogen-induced effector uptake be used as a generic trick to trigger plant immunity?**

Introgression of resistance genes encoding immune receptors recognizing specific effector proteins, into crops by traditional breeding is the most widely used strategy to produce disease resistant plants (Ercolano et al., 2012). A drawback of this approach is the narrow - often race specific resistance - conferred by the resistance protein as it typically recognizes a single effector protein only. This property allows the pathogen to relative easily overcome resistance through mutation or loss of a single effector gene. A prime example for this is the *I-2*-mediated resistance against *Fol* that has been overcome by race 3 isolates through either a single nucleotide substitution or deletion of a single triplet in *Avr2* leading to changes in the Avr2 protein (Houterman et al., 2009; Chellappan et al., 2016). An alterative strategy to breed for resistance is to utilize recessive susceptibility genes that disturb compatibility between pathogen and host. Although this type of resistance is typically non-race specific and expected to be more durable as the pathogen has to gain new traits rather than to lose one, the plant genes involved often play key roles in host processes limiting their utilization potential (van Schie and Takken, 2014). Besides, introgression of genes into crops from wild relatives or from landraces is typically a lengthy and laborious process (van Schie and Takken, 2014). Due to these drawbacks, there is an urgent demand for alternative strategies to introduce disease resistances in crops that are preferably broad spectrum and durable.

The observation that the extracellular effector protein Avr2 is only taken up by plant cells in the presence of a pathogen allows one to exploit this unexpected property of effectors to design a strategy in which plant immunity is triggered by the mere presence of a pathogen. The proposed strategy is to generate transgenic plants producing an extracellularly localized effector protein that matches a cognate Resistance (R) protein that is localized intracellularly. In the absence of a pathogen the intracellular R protein will not perceive the extracellular effector, the immunity response will not be activated and plants develop normal. However, in the presence of a pathogen, effector uptake is triggered, resulting in its recognition by the cognate R protein and subsequent induction of immune responses that will halt further pathogen ingress. The advantage

of such a strategy over classical breeding is that its conferred resistance is potentially more durable as it can only be overcome by pathogens that have lost the ability to induce effector uptake. In practice this essentially means that the pathogen will also have lost its pathogenicity as also its endogenous effectors will no longer be able to enter the host. Another potential advantage of the proposed strategy is that defense activation is expected to occur rapidly upon entry of a pathogen, as the available effector proteins just need to be taken up and no biosynthesis step is involved. This rapid response is expected to restrict the timeframe in which a potential pathogen can interfere with host immune responses by producing and translocating its own effector proteins to counteract host defenses. The proposed strategy might be applicable in a wide variety of plants to confer resistance to all pathogen types that induce effector uptake. In Chapter 4 we have shown that the bacterium *A. tumefaciens* and the fungus *F. oxysporum* trigger effector uptake, but currently it is unknown which other pathogens trigger uptake. Many fungal and oomycete pathogens secrete their effectors in either the apoplast or in confined extracellular spaces, such as the space between the feeding structure of the pathogen that has invaginated the plant cell wall, but not its plasma membrane (Panstruga and Dodds, 2009; Dodds and Rathjen, 2010). Yet, many of these effectors function inside host cells, suggesting their uptake by the plant (Petre and Kamoun, 2014). Examples of such pathogens are the fungi *M. lini* and *Magnaporthe grisea* or oomycetes such as *P. infestans* or *Peronospora* species. It is currently unknown whether uptake of these effectors also requires the presence of the pathogen, but if so, the proposed strategy will likely be applicable to confer resistance to these organisms. If it is merely the presence of non-self molecules inside the plant that trigger effector uptake then the proposed strategy might also confer resistance to bacterial pathogens and potentially intracellular pathogens such as viruses and phytoplasms as well. Future experiments, using the materials described in Chapter 4, will allow one to test whether these foreign entities do trigger effector uptake. It will also be interesting to assess whether beneficial microbes, such as mycorrhiza or endophytes can induce uptake, which obviously would limit the applicability of the proposed strategy.

Another limitation of the applicability of the proposed strategy is that a matching pair consisting of an extracellular Avr protein and an intracellular immune receptor is required. Hence, not all available R/Avr pairs can be used as many of these do not fit these requirements. Besides Avr2 and I-2, R/Avr pairs that fit the requirements and are good candidates to assess feasibility of this concept are for instance the L/AvrL, M/AvrM, R3a/Avr3a, Avr-Pita/Pita or ATR1/RPP1 pairs from flax, flax, potato, rice and Arabidopsis, respectively (Orbach et al., 2000; Dodds et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005; Catanzariti et al., 2006). The intracellular R proteins L and M in flax (*Linum usitatissimum*) mediate recognition of the secreted effector proteins AvrL and AvrM from *M. lini*, respectively (Dodds et al., 2006). Avr3a from *P. infestans* is recognized

in the host cytoplasm, where it triggers R3a-dependent cell death in potato (Armstrong et al., 2005). *Magnaporthe oryzae* effector protein AVR-Pita is predicted to bind directly to the cognate Pita protein inside a plant cell to initiate hypersensitive resistance in rice (Jia et al., 2000). ATR1 is secreted by the Arabidopsis pathogen *Hyaloperonospora arabidopsidis* and recognized specifically by intracellular protein RPP1 (Fabro et al., 2011). In the examples above effector proteins are used that besides an avirulence activity also exert an intrinsic virulence function. Although this latter activity will not be manifested when the protein is located outside the cell, it could interfere with the induction of a full immune response. Hence the use of an effector protein is preferred that retains its avirulence activity, but is mutated in its virulence function. In this thesis we show that for the Avr2 protein of *Fol* it is possible to uncouple these traits, making these mutants prime candidates to employ in the proposed strategy.

**How does Avr2 exert its virulence function, how does it suppress the PTI response?**

As presented in Chapter 5, *ΔspAvr2* transgenic tomato plants are hyper-susceptible to various pathogens, including *Verticillium dahliae*, *Pseudomonas syringae* and *Botrytis cinerea*. Moreover, *ΔspAvr2* transgenic tomato plants are attenuated in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI): flg22-induced growth inhibition, reactive oxygen species (ROS) production and callose deposition are greatly alleviated in *ΔspAvr2* plants. Exactly how Avr2 subverts flg22-induced PTI and contributes to hyper susceptibility to the various pathogens is unknown.

Plants activate their immune systems in response to specific elicitors produced by pathogens (Chisholm et al., 2006; Jones and Dangl, 2006). Many of these elicitors constitute conserved PAMPs, which can be recognized by pattern recognition receptors (PRRs) present at the plant cell surface. PAMP recognition by a corresponding PRR initiates the PTI response (Jones and Dangl, 2006; Bent and Mackey, 2007; Boller and Felix, 2009; Dodds and Rathjen, 2010; Schwessinger and Ronald, 2012)(Figure 1B). One of the best-studied PAMPs is flg22, a conserved 22-amino acid N-terminal sequence derived from *Pseudomonas aeruginosa* flagellin (Zipfel et al., 2004). PTI responses entail a complex network of signaling pathways. An overview of these can be found in recent reviews (Thomma et al., 2011; Bigeard et al., 2015). A well-known pattern recognition receptor (PRR) in Arabidopsis is the LRR receptor kinase Flagellin Sensing 2 (FLS2), which contains an extracellular leucine-rich repeat (LRR) domain, a transmembrane domain and a cytoplasmic kinase domain (Chinchilla et al., 2006). FLS2 together with the co-receptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) recognizes flg22. Flg22 treatment induces rapid FLS2-BAK1 receptor complex formation at the plasma membrane (Chinchilla et al., 2007; Heese et al., 2007; Schulze et al., 2010) (Figure 1B). Additionally, upon PAMP binding the cytoplasmic kinases Botrytis-induced kinase

1 (BIK1) and the related PBL (PBS1-like) kinase associate with FLS2 and become phosphorylated and are subsequently released from the PRR complex (Lu et al., 2010; Zhang et al., 2010). Upon PAMP perception by PRRs, the earliest known responses, that occur within a few minutes, include influx of extracellular $Ca^{2+}$ in the cytosol, generation ROS and activation of mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs). (Nuhse et al., 2007; Ranf et al., 2011). Deposition of callose, inhibition of plant growth and induction of defense-related genes are late responses that become apparent within days (Boller and Felix, 2009).

So far, the identity of the Avr2 host-target(s) is/are unknown. Given the observation that Avr2 suppresses both early (ROS burst) and late PTI response (growth inhibition and callose deposition), it is conceivable that Avr2 targets an early step in PTI signaling, possibly components of the PRR complexes such as the PRR itself, their co-receptor BAK1, or PRR-BAK1 substrates such as BIK1 and PBL1 (Chinchilla et al., 2007; Veronese et al., 2006; Shi et al., 2013) (Figure 1B). Taking into account that *ΔspAvr2* tomato plants are hyper-susceptible to many different types of pathogens, which are recognized by

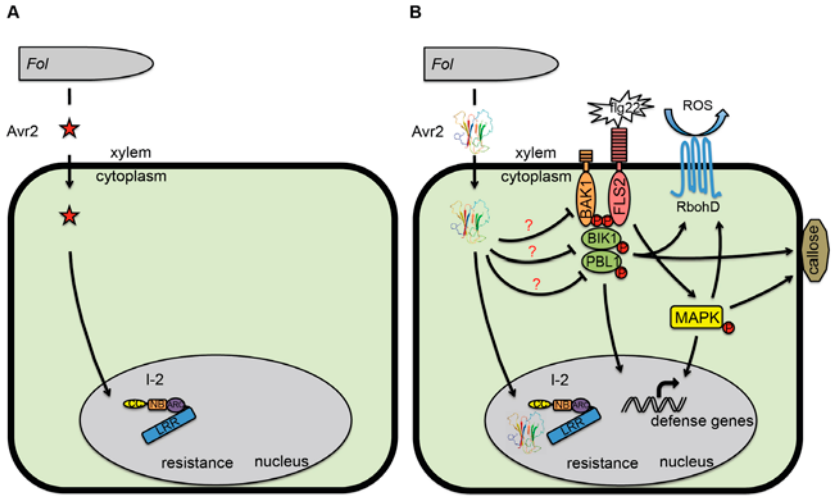


**Figure 1. An updated model for the molecular functions of Avr2 in tomato.** (A) *Fol* secretes Avr2 into the xylem vessels and apoplastic spaces of tomato plants. Avr2 acts inside the plant cell to exert its virulence function. In a resistant plant, *I-2* containing plant immune responses are triggered upon recognition of nuclear-localized Avr2 by I-2. (B) A working model depicting where and how Avr2 exerts its virulence and avirulence function. Avr2 is secreted by *Fol* into the xylem sap and is taken up by plant cells by an unknown mechanism. Avr2 suppresses both flg22 induced early (ROS burst) and late PTI response (callose deposition), implying that Avr2 might target an early step in PTI signaling, possibly positive regulators of PRR complexes such as PRR co-receptor BAK1 or PRR-BAK1 substrates such as BIK1 and PBL1. Additionally, Avr2 can enter the nucleus to active I-2 mediated immune response. (BAK1, BRI1-Associated Receptor Kinase 1; FLS2, Flagellin Sensing 2; BIK1, Botrytis-Induced Kinase 1; PBL1, PBS1-like kinase 1; RbohD, Respiratory burst oxidase homolog D; MAPK, Mitogen-Activated Protein Kinases; ROS, Reactive Oxygen Species; CC, coiled coil; NB, Nucleotide-Binding; ARC, adaptor shared by APAF-1, R proteins and CED-4; LRR, Leucine-Rich Repeat.)

diverse PRRs, it is unlikely that Avr2 targets one specific PRR receptor. Hence, other components are more likely to represent putative Avr2 targets. Previously, activated BIK1 and PBL1 have been shown to phosphorylate and thereby activate the plasma membrane-localized NADPH oxidase, named respiratory burst oxidase homolog D (RbohD), which is responsible for ROS production following PAMP perception. These kinase activities are directly linked to the initiation of ROS production, but they are not required for MPK activation (Zhang et al., 2010; Feng et al., 2012). Other studies showed that MAPK signaling, and then specifically the two MAPKs MPK3 and MPK6, act upstream of RbohD and callose accumulation (Zhang et al., 2007). We therefore reason that it is unlikely that Avr2 targets BIK1 and PBL1 as besides ROS production also callose deposition was suppressed by the presence of Avr2. Excluding these kinases as target of Avr2 points to BAK1 as most likely candidate to be targeted by Avr2. To determine whether Avr2 indeed targets BAK1, one could examine whether accumulation or activity of BAK1 is altered in the presence of Avr2.

Avr2 has structural homology with three distinct proteins, notably the human E3 ubiquitin protein ligase 1 (SIAH1) (Protein Data Bank (PDB) code: 4×3g), the tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) (PDB code: 4z8m) and the MATH-(meprin and TRAF-C homology) domain of Speckle-type POZ (SPOP) protein (PDB code: 3hq1). Ubiquitin is a highly conserved protein found in all eukaryotes and plays important roles in almost all aspects of cell biology, including cell division, growth, communication/signaling, movement and death/apoptosis (Johnson, 2002). Ubiquitination is an important posttranslational modification regulating cellular functions of targeted proteins, including their degradation, trafficking and subcellular localization (Zhou et al., 2014). Ubiquitination consists of a stepwise reaction catalyzed by a series of enzymes including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) (Weissman, 2001). In the initial step, ubiquitin is activated by E1 and then transferred to a Cys residue in E2. E3 subsequently binds both E2 and a target protein and directly or indirectly catalyzes its ligation to ubiquitin. Thus, E3 enzymes are key factors determining substrate specificity by selecting the target proteins for ubiquitination (Weissman, 2001). Various reports show that ubiquitination plays an important role in plant immune responses mediated by both cell surface PRRs and intracellular R proteins (Dielen et al., 2010, Cheng & Li, 2012, Marino et al., 2012). For instance, the two plant U-box E3 ubiquitin ligases PUB12 and PUB13 directly ubiquitinate FLS2 and promote flagellin-induced FLS2 degradation to suppress immune responses (Zhou et al., 2014).

Residues T53 and T145 in Avr2 were identified as critical residues for the virulence function as $Avr2^{T53R}$ and $Avr2^{T145E/K}$ failed to suppress the ROS burst following flg22 treatment. Residues T53 and T145 were chosen for mutagenesis as they are

synonymous to residues in SIAH1 and TRAF6 that are essential for the interaction with partner proteins. The structural homology and conserved functional requirement of the residues indicate that Avr2 might act itself as an ubiquitin ligase and target proteins for ubiquitination and subsequent degradation. If so, it is tentative to speculate that Avr2 might interfere with PTI signaling by promoting ubiquitination of BAK1, thereby down-regulating downstream signaling. *In vitro* assays testing E3 ligase activity of Avr2 on BAK1 might be done to test this hypothesis. Alternatively, Avr2 could interfere with the activity of plant ubiquitin ligases by competing for substrate binding, thereby preventing the ubquitination of important regulators of PTI signaling.

**How does Avr2 trigger I-2-mediated immune response?**

It is unknown how Avr2 is perceived by I-2; its recognition could be direct, a physical interaction of I-2 with Avr2, or indirect via an intermediate host protein. So far, no direct interaction of Avr2 withI-2 could be shown neither in yeast-two-hybrid experiments nor *in planta* (Ma, 2012). The crystal structure of Avr2 showed that the residues in $Avr2^{V41M}$, $Avr2^{R45H}$, $Avr2^{R46P}$ and $Avr2^{T50-}$ (Houterman et al., 2009; Chellappan et al., 2016) required for I-2 recognition form a distinct epitope and cluster at an extended loop. Natural selection resulted in Avr2 mutants that retained virulence, but lost avirulence (Houterman et al., 2009). As mentioned before, structure-guided mutagenesis of Avr2 identified two threonine residues (T53 and T145) whose mutation results in an effector that lost virulence, but retained avirulence. Hence, the virulence and avirulence functions are distinct activities that can be uncoupled and the residues involved in these processes are not overlapping. Therefore, it is likely that I-2-mediated recognition of Avr2 is a direct event as its virulence function is apparently not required for its recognition. Recognition of Avr2 might require only the epitope on Avr2 itself, but could also involve the proximity of two epitopes; one in Avr2 and the other one on its interacting host target. The latter hypothesis can be addressed once the Avr2 host target has been identified, as one would expect that the T53 and T145 mutants retain their ability to interact with this target. If these mutant no longer interact, this would imply that Avr2 alone is required and sufficient for I-2-mediated recognition.

**Concluding remarks and outlook**

In this thesis, a tentative model is proposed depicting how and where Avr2 exerts its virulence and avirulence functions in the cell. We show that besides its avirulence function also its virulence activity requires a cytosolic localization of the protein. The structure of Avr2 allowed us to generate mutants in which avirulence and virulence activity of the protein are uncoupled. The observed structural homology to E3 ubiquitin ligases allows one to formulate testable hypotheses about its mode of action in plant

immunity and a putative mechanism on how it manipulates its target. Discovering that plant cells do not take up Avr2 in the absence of a pathogen provided leads to propose a new strategy for crop protection. This strategy holds great promise to introduce immunity in various plant species to a wide variety of pathogens. A further molecular understanding of the functioning of Avr2 and its recognition by I-2 awaits identification of Avr2 host targets.

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