Multiple faces of Fusarium oxysporum effector protein Avr2

Di, X.

Chapter 1

General introduction

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Vascular wilt disease and its control

Vascular wilt diseases caused by soil-borne fungi are among the most devastating plant diseases worldwide (Tjamos and Beckman, 1989). After penetrating the root and colonizing the vasculature, the pathogen is shielded from most fungicides prohibiting curative treatments of infected plants. Due to the presence of persistent resting structures, such as chlamydospores, vascular fungi exhibit extreme longevity in the soil making crop-rotation futile (Michielse and Rep, 2009). One control measure is soil sterilization, which is expensive and harmful to the soil, making it applicable only on small scales such as in greenhouses. An alternative method of disease control is the use of resistant plant varieties generated by plant breeding. However, when resistances are introduced into elite varieties, they are often overcome by the pathogen in the course of time (Michielse and Rep, 2009). The high economic impact of wilt diseases, combined with the lack of sustainable control treatments, substantiates the need for alternative control strategies. To design novel strategies a thorough understanding of the molecular mechanism underlying disease-susceptibility and -resistance to vascular pathogens is of fundamental importance and the main aim of this thesis is to contribute to this.

Plant innate immune response

To defend themselves against pathogens plants rely on a sophisticated innate immune system (de Wit, 2007). Two distinct layers can be distinguished: the first is based on the perception of conserved pathogen-associated molecular patterns (PAMPs) from pathogens by host pattern-recognition receptors (PRR) (Nurnberger et al., 2004; Zipfel and Felix, 2005). One of the best-characterized PAMP/PRR pair is the FLAGELLIN SENSING 2 (FLS2) receptor mediating recognition of bacterial flagellin (or the elicitor-active peptide flg22 derived from it). PAMP recognition and subsequent activation of the PRR, triggers a basal defence response (also called pattern-triggered immunity - PTI) that wards off attacks by most non-adapted pathogens (Jones and Dangl, 2006). Adapted pathogens can evade or subvert PTI by secretion of so-called "effector" proteins that manipulate host-targets involved in basal defence (Dodds and Rathjen, 2010). Some plants however, carry genes encoding "resistance" proteins that recognise specific effectors allowing them to initiate a second layer of inducible defences, which is called effector-triggered immunity (ETI) (Jones and Dangl, 2006). Pathogens that overcome ETI have often shed, or mutated, the cognate effector thereby evading recognition by the corresponding resistance (R) protein. The outcomes of ETI include an oxidative burst, production of anti-microbial compounds, expression of pathogenesis-related (PR) genes, and often a rapid programmed localized cell death (PCD) response

at the site of infection (Spoel and Dong, 2012). The ETI reaction as a whole is denoted the hypersensitive response (HR) (Mur et al., 2008; Hofius et al., 2011). Notably, the defense responses that are activated during ETI and PTI are partly overlapping, including alterations of phytohormone levels, production of reactive oxygen species (ROS), changes in intracellular calcium levels, transcriptional reprogramming and synthesis of antimicrobial compounds (Coll et al., 2011).

The *Fusarium*-tomato pathosystem

Fusarium oxysporum (*Fo*) is a ubiquitous occurring fungus that inhabits the soil and rhizosphere or even colonizes and lives within plant tissues without causing disease symptoms (Swarupa et al., 2014). Actually, many *Fo* strains confer beneficial effects to their host (Alabouvette et al., 2009; Edel-Hermann et al., 2015; Imazaki and Kadota, 2015). In these cases *Fo* colonization retains symptomless and has beneficial effects on plant growth and/or tolerance to biotic and abiotic stresses. However, a small number of forms evolved into pathogens that cause disease on specific host plants (Michielse and Rep, 2009). In many of these pathogenic interactions a lengthy asymptomatic phase precedes disease development (Demers et al., 2015). Thus, based on its lifestyle pathogenic *Fo* strains are typically classified as hemi-biotrophs, shifting from a biotrophic phase early in infection to necrotrophy at later stages. These pathogenic strains typically produce toxins at later stages of disease development in order to kill the host cells, as to complete their life cycle on dead tissues (Horbach et al., 2011). Pathogenic *Fo* stains have been classified in over 120 formae speciales, which refer to their respective plant hosts, as a particular *forma specialis* (f.sp.) typically produces disease in one or a limited range of host species only (Armstrong and Armstrong, 1981; Katan and Di Primo, 1999). Over past decades the interaction between *Fo* f.sp. *lycopersici* (*Fol*) and tomato (*Solanum lycopersicum*) has evolved into an excellent model to study the molecular mechanisms underlying disease and resistance (Takken and Rep, 2010). Upon recognition *Fol* attaches to the roots, colonizes the cortex and subsequently penetrates the endodermis. It then rapidly colonizes the xylem vessels and eventually the xylem-adjacent tissues (di Pietro et al., 2003). Successful infection causes typical disease symptoms such as yellowing, wilting, stunted growth and in some cases plant death (di Pietro et al., 2003; Agrios, 2005).

Phytohormones such as salicylic acid (SA), ethylene (ET), jasmonic acid (JA), abscisic acid (ABA) and auxin are signaling molecules that are not only essential for regulation of plant growth, development and reproduction, but they also play a vital role in adaptive responses to a wide variety of biotic and abiotic stresses (Bari and Jones, 2009; Grant and Jones, 2009; Pieterse et al., 2009; Pieterse et al., 2012). Plants typically respond to pathogen infection with a complex scenario of sequential, antagonistic or synergistic

action of different hormone signals (Robert-Seilaniantz et al., 2011). In *Arabidopsis thaliana*, various mutants and transgenic lines impaired in hormone biosynthesis, perception or signaling display a severe alteration in their level of susceptibility to *Fo* (Berrocal-Lobo and Molina, 2008; Pieterse et al., 2009; Thatcher et al., 2009; Trusov et al., 2009). For instance, Arabidopsis *NahG* (containing a bacterial salicylate hydroxylase transgene) and *sid2* (salicylic acid induction deficient 2) lines, in which SA accumulation is impaired, exert increased susceptibility to *Fo* f.sp. *conglutinans* (*Focn*) pointing at the involvement of SA in disease susceptibility (Berrocal-Lobo and Molina, 2004; Diener and Ausubel, 2005). ET is also involved in susceptibly, but its role is opposite to that of SA. Arabidopsis mutants *ein2* (ethylene insensitive 2) and *etr1-1* (ethylene receptor 1), both ET insensitive, show a reduction in disease symptoms as compared to Col-0 plants, when inoculated with *Focn* (Trusov et al., 2009; Pantelides et al., 2013). The involvement of phytohormones in plant susceptibility following *Fo* infection has mostly been studied in the model *Arabidopsis thaliana*. Their role in modulating susceptibility of crops, such as tomato, in relation to *Fo* infection has not systematically been studied yet. As their activity can be different in different plant species, it is worthwhile to investigate involvement of these hormones in the *Fol*-tomato pathosystem.

Fol effectors and tomato R proteins

During colonization of its host *Fol* secretes many small proteins into the xylem sap (Rep, 2005). Using mass-spectrometry our lab identified 14 putative effector proteins in the xylem sap of infected susceptible tomato plants, named "Secreted In Xylem" (SIX) proteins (Rep et al., 2005; Houterman et al., 2007; Houterman et al., 2009; Schmidt et al., 2013). For Six1, Six3, Six5 and Six6 a virulence function has been revealed (Rep et al., 2005; Houterman et al., 2009; Gawehns et al., 2014; Ma et al., 2015). Besides, some effectors have been found to act as avirulence factors, triggering resistance gene-mediated immunity in tomato. The resistance thus brought about, fits the "gene-for-gene" model, in which a plant *R* gene requires a "matching" avirulence (*Avr*) gene in the pathogen (Figure 1). So far, three *R* genes against *Fol* have been introgressed into commercially cultivated tomato varieties: genes *I* and *I-2* from *S. pimpinellifolium*, conferring resistance against *Fol* races 1 and 2, respectively, and the *I-3* gene from *S. pennellii*, which confers resistance to *Fol* race 3 (Huang and Lindhout, 1997). Recently, all these three *R* genes have been cloned (Simons et al., 1998; Catanzariti et al., 2015; Catanzariti et al., 2016). The three genes encoding the *Fol* effector proteins Six4 (Avr1), Six3 (Avr2) and Six1 (Avr3), which are recognized by R proteins I, I-2 and I-3, respectively, had already been cloned before (Rep et al., 2004; Houterman et al., 2008; Houterman et al., 2009). The *Avr3* gene encodes a 32kDa protein, which includes a 2kDa signal peptide, an 8kDa N-terminal prodomain and a 22kDa mature protein that represents the C-terminal part of the protein (Rep et al., 2005; van der Does et al., 2008). *Avr2*

encodes a small 15.7kDa protein that is processed after cleavage of its signal peptide (Houterman et al., 2007; Houterman et al., 2009). *Avr3* is expressed when the fungus is in contact with living plant cells (van der Does et al., 2008), while *Avr2* is predominantly expressed in xylem-colonizing hyphae and to some extend in hyphae that reside in the root cortex (Ma et al., 2013). Both Avr3 and Avr2 contribute to pathogenicity as deletion of either *Avr2* or *Avr3* in *Fol* greatly reduced virulence of the knockout strains on susceptible tomato plants (Rep et al., 2005; Houterman et al., 2009). Like *Avr3*, the *Avr1* gene encodes a 27kDa product with a signal peptide and a predicated prodomain (Houterman et al., 2007; Houterman et al., 2008). After processing a 20kDa mature protein remains. Notably, Avr1 does not contribute to fungal virulence on susceptible plants, but it suppresses *I-2* and *I-3* mediated resistance allowing the fungus to cause disease on plants carrying the cognate resistance genes (Houterman et al., 2008). None of the three Avr proteins shares significant homology to any known protein and their mode of actions in the host are unknown (Fraser-Smith et al., 2014).

Avr2 is expressed by the fungus following penetration of the root cortex and during colonization of the xylem vessels in which the encoded protein can be readily detected (Houterman et al., 2009; Ma et al., 2013). *I-2* encodes a coiled coil (CC), nucleotide-binding (NB), ARC domain (for Apaf1, R proteins, and CED4) and leucine-rich repeat (LRR) protein (Simons et al., 1998). Avr2 is recognized inside plant cells by I-2 and a nuclear localization is required to trigger I-2-dependent cell death (Ma et al., 2013) (Figure 1). *Avr2* is found in all *Fol* isolates. However, race 3 isolates carry specific point mutations in *Avr2* or a deletion of one triplet ($Avr2^{V41M}$, $Avr2^{R45H}$, $Avr2^{R46P}$ and $Avr2^{T50-}$, respectively) that allows *Fol* to overcome *I-2* mediated resistance (Houterman et al., 2009; Chellappan et al., 2016). Interestingly, the point mutations neither the deletion in the race 3 *Avr2* variants do negatively affect virulence of race 3 *Fol* strains, which shows that avirulence of Avr2 can be uncoupled from its virulence function (Houterman et al., 2009; Chellappan et al., 2016).

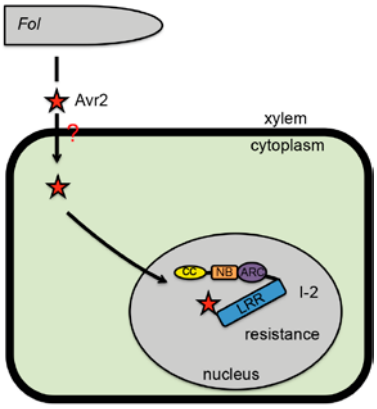

Figure 1. A schematic representation of the "gene-for-gene" resistance in the *Fusarium*-tomato interaction. *Fol* secretes Avr2 into the xylem vessels and apoplastic spaces of tomato plants. Subsequently, it is translocated into the cell by a hitherto unknown mechanism. Avr2 acts inside the plant cell 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.

I-2 is specifically expressed in the parenchyma cells adjacent to the xylem vessels (Mes et al., 2000). Currently it is unknown how Avr2 present in the xylem sap is translocated into *I-2* expressing plant cells allowing its perception by the host. *I-2* mediated resistance does not lead to a typical HR response in infected roots, as cell death is not induced (Beckman, 2000). Nevertheless, *I-2* can induce cell death upon the systemic expression of *Avr2* using a Potato virus X (PVX)-based expression system or when *Avr2* and *I-2* are transiently co-expressed in leaves of the non-host *Nicotiana benthamiana* (Houterman et al., 2008; Houterman et al., 2009; Ma et al., 2013). The well-characterized *Avr2-I-2* gene pair makes the *Fol*-tomato interaction a perfect model to study the molecular basis of disease and resistance to wilt diseases.

Outline of the thesis

In **Chapter 2**, the current knowledge on the role of the major phytohormones including SA, JA, ET, ABA and auxin on the interaction between *Fo* and its diverse hosts is summarized. We discuss how phytohormones determine the *Fo* lifestyle, and how phytohormones and *Fo* effectors together act to control the balance between a beneficial and a pathogenic interaction and *vice versa*. **Chapter 3** investigated the role of SA, JA and ET in tomato susceptibility to *Fol*. Thereto, mutants affected in the biosynthesis and perception of SA, ET and JA were inoculated with wild-type *Fol* or a less pathogenic mutant derived from *Fol007* in which the *Avr2* gene was deleted (*FolΔAvr2*). Finally, a model for the role of SA, ET and JA signaling in the susceptibility of tomato against *Fol* is presented and compared to the involvement of these hormones in the susceptibility of Arabidopsis plant to *Focn*. In **Chapter 4** the virulence function of Avr2 was investigated. To identify whether Avr2 acts inide or outside host cells, transgenic tomato plants stably expressing full-length *Avr2* or a cytosolic *Avr2* (*ΔspAvr2*) variant that lacks the signal peptide for secretion were generated. The observation that pathogenicity of an *Avr2* knockout *Fusarium* (*FolΔAvr2*) strain was fully complemented in *ΔspAvr2* transgenic tomato lines suggests that Avr2 exerts its virulence functions inside host cells. This hypothesis is in line with the observation that Avr2 recognition by I-2 occurs inside plant cells (Ma et al., 2013). Surprisingly, plant-produced full length Avr2 can also fully complement *FolΔAvr2* virulence. Western blot analysis revealed that Avr2, in contrast to ΔspAvr2, was readily detected in the xylem sap and apoplastic fluids, implying that Avr2 is taken up by plant cells from the extracellular spaces. Additional experiments using other pathogens revealed that Avr2 uptake is not a host autonomous event and relies on the presence of a pathogen. In **Chapter 5** it is shown that tomato plants expressing *ΔspAvr2* become hyper-susceptible towards various plant pathogens, such as *Fusarium oxysporum*, *Verticillium dahliae* and *Pseudomonas syringae*. This observation prompted us to examine whether Avr2 specifically interferes with PTI responses. Moreover, to understand how Avr2 functions at a molecular level its crystal structure was solved in

collaboration with M. Banfield at the John Innes Centre, UK. The structural homology that Avr2 shares with some proteins with known activities allowed prediction of residues that might be involved in the interaction of Avr2 with partner proteins. Two of these putative important residues were found to be required for virulence activity of the protein and enabled us to uncouple virulence from avirulence function. The results described in this thesis are summarized and discussed in **Chapter 6**.

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