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### The role of *Fusarium oxysporum* effector protein Avr2 in resistance and pathogenicity

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# **CHAPTER 1**

## **General introduction and outline**

## Introduction

Plants are continuously exposed to microorganisms. In their attempts to acquire nutrients pathogenic microorganisms invade various plant tissues, including underground tissues such as roots. Root-infecting pathogens that are most widely distributed in soil encompass fungi, oomycetes, bacteria and nematodes. Soil-born pathogenic fungi comprise distinct genera, such as *Fusarium*, *Rhizoctonia*, *Sclerotinia*, *Thielaviopsis*, and *Verticillium*. Within the genera *Fusarium* and *Verticillium* many species are found that cause vascular wilt diseases on economically important plants. Examples of such species are *Fusarium oxysporum*, *Verticillium dahliae* and *Verticillium albo-atrum* (Gordon and Martyn, 1997; Klosterman *et al.*, 2009; Singleton *et al.*, 1992; Tjamos and Beckman, 1986). *Fusarium oxysporum* can infect over 100 different plant species, and based on its host specificity the species is subdivided into host-specific forms, called *formae speciales* (f.sp.) (Armstrong and Armstrong, 1981; Di Pietro *et al.*, 2003).

The main measures to control vascular diseases caused by soil-born fungi are soil fumigation and application of resistant cultivars (Beckman, 1987; Fravel *et al.*, 2003). Application of first control measure is increasingly restricted due to negative effects on the environment and the health risks for humans. Hence, the use of resistant crop cultivars is the solely remaining alternative to control vascular diseases. Unfortunately only for some plant species natural resistances are available for introgression, and most often these are monogenic traits. The ability of individual *formae speciales* of *F. oxysporum* to infect specific plant cultivars carrying such a resistance gene, is used to further subdivide these *formae speciales* into specific races (Di Pietro *et al.*, 2003; Michielse and Rep, 2009). The monogenic resistances conferred by resistance genes can be broken by the pathogen, as it often requires only a single mutation in a corresponding avirulence gene of the pathogen to evade recognition by the host. Whereas resistance genes can quickly be broken, their introgression into cultivars is often a time-consuming process (Fravel *et al.*, 2003; Gordon and Martyn, 1997). This discrepancy stresses the urge to develop new and alternative means to control these devastating diseases. To develop such strategies a solid understanding of the molecular mechanisms underlying compatible and incompatible interactions between vascular pathogens and their hosts is needed.

The *F. oxysporum*-tomato interaction has shown to represent an excellent system to study - at the molecular level - the interaction between a vascular fungus and its host (Takken and Rep, 2010). In this thesis I focus on the molecular interaction between tomato and the wilt causing fungus *F. oxysporum* f.sp. *lycopersici*. In the next sections, I describe the infection process of *F. oxysporum* f.sp. *lycopersici*, its effector proteins and the corresponding tomato resistance genes.

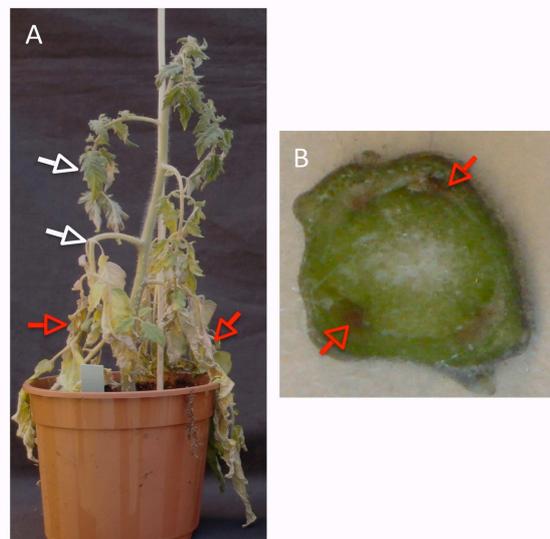
### **Infection process of *F. oxysporum* f.sp. *lycopersici* on tomato**

*F. oxysporum* f.sp. *lycopersici* (*Fol*), the causal agent of Fusarium wilt of tomato (Figure 1), is assumed to reproduce asexually as no sexual stage has ever been observed. For its reproduction the fungus produces three types of spores with distinct morphological features; macroconidia containing three to five cells, kidney-shaped microconidia and the thick-walled chlamydospores (Gordon and Martyn, 1997). As a soil inhabitant, *Fol* can survive for extended periods of time in the form of chlamydospores (Di Pietro *et al.*, 2003). The infection process of *Fol* in tomato plants has been studied extensively using light, confocal and electron microscopy. These studies were focused on different stages of infection, such as root recognition, root surface attachment and colonization, penetration and colonization of root cortex, hyphal proliferation and production of microconidia within xylem vessels (Di Pietro *et al.*, 2003; Michielse and Rep, 2009; Olivain *et al.*, 2006). Upon contact with a tomato root, germinated spores form hyphal threads that can enter the root through natural wounds or by direct penetration of the epidermal cell walls (Figure 2)(Lagopodi *et al.*, 2002; Rodriguezgalvez and Mendgen, 1995). After colonization of the cortex, *Fol* enters the xylem vessel (Figure 2) in which it proliferates rapidly to spread throughout the vessel. The consequence of the accumulation of fungal biomass in the xylem vessel together with the local host defense responses, such as the formation of tyloses and the production of gels and gums, result in clogging of the vessels (Beckman, 1987; Beckman, 2000; Olivain *et al.*, 2006). Clogging of the xylem vessels is regarded as a key reason for disturbed water transport to the foliage, leading to the typical wilt symptoms of an infected tomato plant. During later stages of infection *Fol* invades the parenchyma cells adjacent to the xylem vessels, and colonizes neighboring non-infected vessels in the tomato root and stems. Finally, the fungus sporulates on the dead plant tissues thereby completing its lifecycle (Di Pietro *et al.*, 2003). Because *Fol* kills its host at late stages of infection, it is commonly considered a hemibiotrophic pathogen.

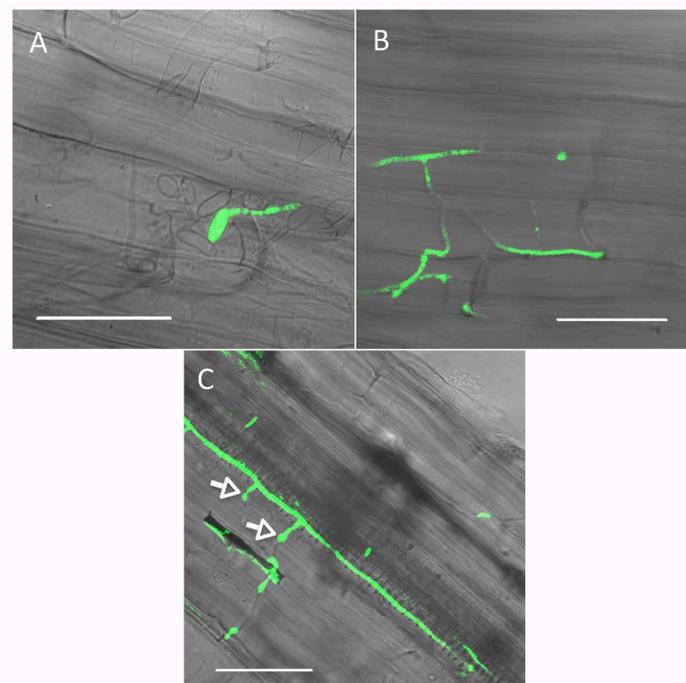
### **Resistance genes against *Fol* in tomato**

Some wild tomato relatives carry natural resistance against specific races of *Fol*. These resistances are often conferred by monogenic and dominant genes called resistance (*R*)- or immunity (*I*) genes. Some of these *R* genes have been introgressed into commercial tomato cultivars and based on the order in which they have been introgressed they have been named *I* or *I-1*, *I-2* and *I-3*. The *R* genes confer race specific resistance, which implies that the *Fol*-tomato interaction complies with the gene-for-gene hypothesis that states that for each plant *R* gene the pathogen carries a corresponding avirulence (*AVR*) gene. Of the three *R* genes only *I-2* and *I-3* have been cloned. *I-2* is predicted to encode an intracellular protein from the CC-NB-LRR family whereas *I-3* encodes SRLK-receptor like kinase ((Simons *et al.*, 1998)

and Ann-Maree Catanzariti, personal communication). NB-LRR proteins contain a nucleotide-binding domain fused to a leucine-rich repeat region. On their N-terminus these

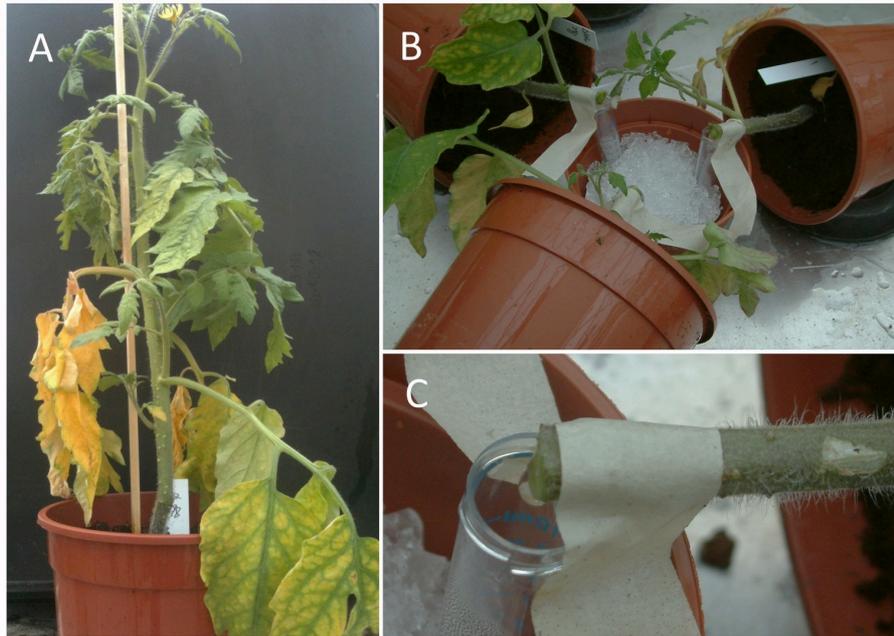


**Figure 1. Typical disease symptoms on tomato plants infected by *F. oxysporum* f.sp. *lycopersici* (*Fol*).** **A)** An infected plant showing severe wilting symptoms. Four-week-old tomato plants (cultivar moneymaker C32) were inoculated with a *Fol* isolate and a representative plant was photographed 3-week-post inoculation. The white arrows indicate the epinastic response seen in young petioles and leaves, and the red arrows indicate the chlorosis and desiccation observed in older leaves. **B)** A cross-section of the stem of an infected tomato plant showing the browning of the xylem vessels, which is indicated with a red arrow.



**Figure 2. Sequential infection steps of *Fol* on susceptible tomato roots.** Tomato roots inoculated with a *Fol* strain expressing green fluorescent protein (GFP) under the control of the *SIX1* promoter. Scale bar represents 25  $\mu\text{m}$  in length. **A)** The infection hypha of a germinated microconidium penetrating the root epidermis. **B)** Invasive hyphae colonizing the root cortex. **C)** Hyphae growing inside the xylem vessel and in some cases they are able to colonize the adjacent cells (arrows).

proteins often carry a domain that either resembles a coiled-coil domain, as is the case for *I-2*, or a domain with homology to the Toll/interleukin-1 receptor (TIR) (Simons *et al.*, 1998; Takken and Tameling, 2009). Studies using the *I-2* promoter fused to the  $\beta$ -glucuronidase (*GUS*) reporter gene showed that *I-2* is specifically expressed in the parenchyma cells adjacent to the xylem vessels. *Fol* does not colonize the xylem parenchyma cells in a resistant *I-2* plant, suggesting that these cells mediate *I-2* specific resistance (Mes *et al.*, 2000).



**Figure 3. Collection of xylem sap from *Fol* infected tomato plants.** **A)** Typical disease symptoms of an infected tomato plant that is used for xylem sap collection. Four-week-old tomato plants (cultivar moneymaker C32) were inoculated with a *Fol* isolate, the picture was taken two weeks after inoculation. **B)** Harvesting xylem sap. Stems of infected tomato plants were cut below the second true leaf and plants were placed horizontally. Xylem sap bleeding from the stem was collected in a tube placed on ice. **C)** Close-up of a droplet of xylem sap originating from the wound side.

### Effectors and avirulence proteins of *Fol*

Since *Fol* is a xylem-colonizing fungus, the xylem serves as the main interface where host and pathogen interact. To reveal the interactome of *Fol* and tomato, xylem sap has been isolated from infected plants as shown in Figure 2 (Houterman *et al.*, 2007). Eleven small *Fol* proteins (<25 kDa), called SIX (secreted in xylem) proteins, were identified using two-dimensional gel separation followed by mass spectrometric analysis (Houterman *et al.*, 2007; Takken and Rep, 2010). In addition, thirteen tomato derived proteins whose abundance changes in response to *Fol* infection were found. The tomato proteins include glucanases, chitinases and other pathogenesis-related (PR) proteins (Houterman *et al.*, 2007; Rep *et al.*, 2002). Two out of the eleven SIX proteins, notably Six4 (*Avr1*) (Houterman *et al.*, 2008)

and Six1 (Avr3) (Rep *et al.*, 2004) have been demonstrated to represent avirulence proteins recognized by respectively I-1 and I-3. Besides being an avirulence factor Avr3 also was shown to be required for virulence as deletion of *AVR3* compromises pathogenicity of *Fol* on susceptible tomato plants (Rep *et al.*, 2004). Although *AVR1* does not contribute to virulence on a susceptible tomato plant, an *AVR1* knockout loses its pathogenicity on tomato plants containing either the *I-2* or *I-3* gene. The inability of the *AVR1* knockout to overcome these two resistance genes suggests that Avr1 suppresses I-2 and I-3-mediated resistance to promote fungal virulence on resistant plants (Houterman *et al.*, 2008).

## Outline of the thesis

To further unravel the molecular interaction between *Fol* and tomato, this thesis focuses on the interaction between I-2 and its corresponding avirulence protein: Avr2. *I-2* has been cloned over a decade ago and here I will describe the cloning of *AVR2* thereby completing the first gene-for-gene pair of an interaction between a xylem-colonizing fungus and its host. The identification of the *AVR2* gene provided a good starting point to elucidate the molecular basis of the interaction between Avr2 and I-2, and of *I-2*-mediated resistance. In this thesis, the identification of Avr2 and its functional aspects towards *Fol* pathogenicity and *I-2* mediated resistance are described. First the molecular basis of effector recognition by plant NB-LRR protein is reviewed in **Chapter 2**. Three specific topics are discussed including: 1) structural features of subdomains in NB-LRR proteins, 2) assembly of these subdomains into a signaling competent NB-LRR and 3) effector recognition by NB-LRRs and the subsequent induction of defense signaling.

In **Chapter 3**, the characterization of *AVR2* is described. A *SIX3* knockout was found to become pathogenic on *I-2* tomato plants, identifying *SIX3* as *AVR2*. Its ability to activate I-2 was examined using the *Agrobacterium*-mediated transient expression system in *Nicotiana benthamiana* leaves and a PVX-mediated expression system in tomato plants. The mechanism underlying evasion of *I-2*-mediated resistance by *Fol* race 3 isolates was studied by sequence analysis of the *AVR2* gene in these races. Avr2 is present in the xylem sap of infected tomato plants, yet activation of I-2 by Avr2 occurs inside plant cells. This discrepancy suggests that Avr2 is capable of entering the plant cell. In **Chapter 4** the localization of transiently expressed *AVR2* in *N. benthamiana* as well as the location of Avr2 in *Fol*-infected tomato roots is studied using confocal microscopy. Furthermore, a structure-function analysis of Avr2 is performed using gene truncation studies, yeast two-hybrid and co-immunoprecipitation experiments and mass spectrometry analysis.

As part of the ongoing functional analysis of *SIX* genes, a *SIX5* knockout is described in **Chapter 5**. A knockout in *Fol* is created via homologous recombination and the contribution of *SIX5* to virulence and avirulence on *I-2* tomato line was determined in

bioassays. These assays revealed that Six5 and Avr2 form a functional unit that is required for *I-2*-mediated resistance. The underlying mechanism is studied by analyzing the expression of *SIX5* and *AVR2* *in vivo* and by determining the accumulations of these proteins in the xylem sap of infected tomato plants. **Chapter 6** focuses on a widely used technique in plant pathology: Agrobacterium-mediated transient expression (agroinfiltration). This method was optimized and applied for transient expression of *SIX* effectors and *R* genes in the leaves of *N. benthamiana*. This expression system enabled me to study the interactions and subcellular localization of the encoded proteins. The practical details of this method are presented as a technical paper to allow its application in molecular plant research. In **Chapter 7** generation of transgenic Arabidopsis and tomato constitutively expressing *AVR2* is described. Further characterizations of *AVR2*-expressing transgenic plants are provided.

The results described in this thesis are summarized and discussed in **Chapter 8**. A putative model is proposed that describes our current insight in *AVR2/SIX5/I-2*-mediated resistance and the concomitant cell death. Finally, directions for future studies aimed to increase our understanding of the role of Avr2 for infection of tomato are given.

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