Multiple faces of Fusarium oxysporum effector protein Avr2

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CONTENTS

Chapter 1

General introduction

General introduction

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).

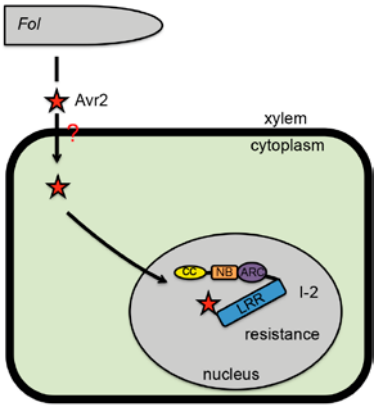

Figure 1. A schematic representation of the "gene-for-gene" resistance in the *Fusarium*-tomato interaction. *Fol* secretes Avr2 into the xylem vessels and apoplastic spaces of tomato plants. Subsequently, it is translocated into the cell by a hitherto unknown mechanism. Avr2 acts inside the plant cell 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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Chapter 2

How phytohormones shape interactions between plants and the soil-borne fungus *Fusarium oxysporum*

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Abstract

Plants interact with a huge variety of soil microbes, ranging from pathogenic to mutualistic. The *Fusarium oxysporum* (*Fo*) species complex consists of ubiquitous soil inhabiting fungi that can infect and cause disease in over 120 different plant species including tomato, banana, cotton and Arabidopsis. However, in many cases *Fo* colonization remains symptomless or even has beneficial effects on plant growth and/or stress tolerance. Also in pathogenic interactions a lengthy asymptomatic phase usually precedes disease development. All this indicates a sophisticated and fine-tuned interaction between *Fo* and its host. The molecular mechanisms underlying this balance are poorly understood. Plant hormone signaling networks emerge as key regulators of plant-microbe interactions in general. In this review we summarize the effects of the major phytohormones on the interaction between *Fo* and its diverse hosts. Generally, Salicylic Acid (SA) signaling reduces plant susceptibility, whereas Jasmonic Acid (JA), Ethylene (ET), Abscisic Acid (ABA) and auxin have complex effects, and are potentially hijacked by *Fo* for host manipulation. Finally, we discuss how plant hormones and *Fo* effectors balance the interaction from beneficial to pathogenic and *vice versa*.

Keywords: plant immunity, root pathogen, vascular wilt disease, effectors, endophyte

Introduction

Fusarium oxysporum (*Fo*), one of the most relevant plant pathogens in global agriculture, is a widespread soil-borne fungus that invades roots and causes vascular wilt disease through colonization of xylem tissue (Tjamos and Beckman, 1989). Pathogenic *Fo* stains have been classified in more than 120 *formae specials* (ff.spp.), which refers to a specific plant host, as a particular isolate typically produces disease only within a limited range of host species (Armstrong and Armstrong, 1981; Katan and Di Primo, 1999) (Table 1). The infection process occurs following attachment to the root surface and subsequent penetration and colonization of the plant root and proliferation within the xylem vessels, leading to both local and systemic induction of a broad spectrum of plant defense responses (Berrocal-Lobo and Molina, 2008). Vascular browning, stunting, progressive wilting and eventually plant death are typical disease symptoms in infected plants (di Pietro et al., 2003; Agrios, 2005). In contrast to the potential of pathogenic *Fo* isolates to cause destructive plant diseases, many *Fo* strains are nonpathogenic and survive either saprophytically in the soil, as non-invasive colonizer of the rhizosphere, or as endophyte inside plant tissues (Kuldau and Yates, 2000; Edel-Hermann et al., 2015; Imazaki and Kadota, 2015). There is relatively little known about the lifestyle strategies of these inconspicuous endophytic strains, but some of them have been successfully employed in biocontrol strategies to combat plant diseases (Alabouvette et al., 2009; Vos et al., 2014).

Table 1. *Fo* strains and their host described in the manuscript.

Abbreviations	formae speciales	Host
Foal	*Fusarium oxysporum* f.sp. *albedinis*	date palm
Focn	*Fusarium oxysporum* f.sp. *conglutinans*	cabbage, Arabidopsis
Focb	*Fusarium oxysporum* f.sp. *cubense*	banana
Fol	*Fusarium oxysporum* f.sp. *lycopersici*	tomato
Fomt	*Fusarium oxysporum* f.sp. *matthioli*	garden stock, Arabidopsis
Foph	*Fusarium oxysporum* f.sp. *phaseoli*	common bean
Forp	*Fusarium oxysporum* f.sp. *raphani*	radish, Arabidopsis
Forl	*Fusarium oxysporum* f.sp. *radicis-lycopersici*	tomato

Based on their lifestyle plant pathogenic fungi have been classified as biotrophs and necrotrophs. Biotrophic pathogens derive nutrients from living cells and deploy complex manipulation strategies to exploit their hosts while keeping them alive. In contrast, necrotrophic pathogens generally kill host cells and feed on their contents, resulting in extensive necrosis, tissue maceration and plant rot (Glazebrook, 2005). A third type, termed hemi-biotrophs, displays both forms of nutrient acquisition, shifting from a biotrophic phase early in infection to necrotrophy at later stages. These pathogens typically produce toxins only at later stages of disease development in order to kill the host cells and to complete their life cycle on dead tissues (Horbach et al., 2011). The

strategy of different pathogenic *Fo* strains can vary, but is usually best described by a hemi-biotrophic lifestyle (Michielse and Rep, 2009). Consistently, the *Fo* genomes show an expansion of genes that encode small, secreted proteins as well as cell-wall degrading enzymes, a feature shared by many hemi-biotrophic fungi (Lo Presti et al., 2015). Analysis of the xylem sap proteome from *Fol*-infected tomato plants identified numerous fungal proteins, termed Secreted in Xylem (Six) protein. For several Six proteins a contribution to virulence has been demonstrated, designating them as *bona fide* effectors (Takken and Rep, 2010; de Sain and Rep, 2015). However, their molecular mode of action and putative virulence targets remain unknown.

Phytohormones such as SA, JA and ET, are known to play major roles in regulating plant defense responses against various pathogens. Generally, SA signaling triggers resistance against biotrophic and hemibiotrophic pathogens, whereas a combination of JA and ET signaling activates resistance against necrotrophic pathogens (Glazebrook, 2005). All these hormones are part of a larger signaling network that integrates environmental inputs and provides robustness against microbial manipulations (Katagiri and Tsuda, 2010; Pieterse et al., 2012). Additional hormones such as auxins, abscisic acid (ABA), gibberellic acids (GAs) and brassinosteroids (BRs) have also been reported to be involved in plant immunity and to fine-tune immunity and growth/development in plants (Table 2) (Robert-Seilaniantz et al., 2011). In this review we summarize the current knowledge on the role of phytohormones in plant disease and resistance triggered by different *Fo* ff. spp. to uncover how they shape the outcome of this widespread plant-fungal interaction.

SA promotes resistance to *Fo*

Defense to biotrophic or hemibiotrophic pathogens is frequently mediated via SA signaling (Glazebrook, 2005). Arabidopsis plants with impaired SA accumulation showed increased susceptibility to *Fo* f.sp. *conglutinans* (*Focn*), but not to f.sp. *raphani* (*Forp*) pointing to an isolate-specific role of SA-dependent defense responses (Table 2)(Berrocal-Lobo and Molina, 2004; Diener and Ausubel, 2005; Trusov et al., 2009; Cole et al., 2014). Interestingly, mutants of the SA master-signaling regulator NPR1 showed wildtype (WT)-like susceptibility to *Focn* when 2-3-week-old soil grown plants were examined (Diener and Ausubel, 2005; Trusov et al., 2009). In contrast, when seedlings were infected on sterile agar plates *npr1* mutants displayed clearly enhanced susceptibility in comparison to WT (Berrocal-Lobo and Molina, 2004). SA accumulation and signaling is also influenced by the nucleo-cytoplasmic proteins PAD4 and EDS1. Soil-grown *pad4*, but not *eds1*, Arabidopsis showed increased susceptibility, whereas sterile grown *pad4* seedlings behaved like WT (Berrocal-Lobo and Molina, 2004; Diener and Ausubel, 2005; Trusov et al., 2009). Thus, the influence of SA signaling regulators

Table 2. Phytohormone mutants involved in the defense response against *Fo* infection.

Hormones	Mutants and transgenic lines	Process affected	Plant species	Susceptibility	References
SA	*NahG*	SA accumulation	Arabidopsis	increased to *Focn* and *Fol*	(Berrocal-Lobo and Molina, 2004; Diener and Ausubel, 2005; Thatcher et al., 2009; Trusov et al., 2009)
	sid2-1	SA biosynthesis	Arabidopsis	increased to *Focn* and *Fol*	(Berrocal-Lobo and Molina, 2004; Diener and Ausubel, 2005)
	eds1-1, eds1-22	SA signaling	Arabidopsis	unaltered to *Focn* and *Fol*	(Berrocal-Lobo and Molina, 2004; Trusov et al., 2009)
	eds3, eds4, eds10	SA signaling	Arabidopsis	increased to *Focn*	(Diener and Ausubel, 2005)
	eds5-1	SA biosynthesis	Arabidopsis	increased to *Focn* and *Fol*	(Berrocal-Lobo and Molina, 2004; Diener and Ausubel, 2005; Thatcher et al., 2009; Trusov et al., 2009)
	pad4-1 *pad4*	SA signaling	Arabidopsis	unaltered to *Focn* and *Fol* increased to *Focn*	(Berrocal-Lobo and Molina, 2004) (Diener and Ausubel, 2005)
	npr1-1	SA perception	Arabidopsis	increased to *Focn* and *Fol*	(Berrocal-Lobo and Molina, 2004)
	npr1-1, npr1-2, npr1-3, npr1-4	SA perception	Arabidopsis	unaltered to *Focn*	(Diener and Ausubel, 2005; Trusov et al., 2009)
	35S::NPR1	SA perception	tomato	reduced to *Fol*	(Lin et al., 2004)
	hpSAMT	SA metabolism	tomato	reduced to *Fol*	(Ament et al., 2010)
JA	*aos, fad3-2, opr3 fad7-1 fad8*	JA biosynthesis	Arabidopsis	unaltered to *Focn*	(Thatcher et al., 2009)
	coi1, coi1-21	JA perception	Arabidopsis	reduced to *Focn* and *Fomt*,	(Thatcher et al., 2009; Trusov et al., 2009; Cole et al., 2014)
	jar1-1 *jar1-1*	JA-Ile biosynthesis	Arabidopsis	increased to *Focn* and *Fol* unaltered to *Focn*	(Berrocal-Lobo and Molina, 2004; Trusov et al., 2009) (Thatcher et al., 2009)
	jin1-9(atmyc2-3), jin1-9/myc2	JA signaling	Arabidopsis	reduced to *Focn*	(Anderson et al., 2004; Trusov et al., 2009)
	35S::AtERF2	Positive regulator of MeJA response	Arabidopsis	reduced to *Focn*	(McGrath et al., 2005)
	35S::AtERF4	Negative regulator of MeJA response	Arabidopsis	increased to *Focn*	(McGrath et al., 2005)
	pft1-1, med8	JA signaling	Arabidopsis	reduced to *Focn*	(Kidd et al., 2009)
	def1	JA biosynthesis	tomato	increased to *Forl* and *Fol*	(Thaler et al., 2004; Kavroulakis et al., 2007)
	jai1	JA perception (Coi1 homolog)	tomato	unaltered to *Fol*	(Cole et al., 2014)
ET	*ein2-1*	ET signaling	Arabidopsis	reduced to *Focn* and *Forp*	(Trusov et al., 2009; Cole et al., 2014)
	ein2, etr1	ET signaling	Arabidopsis	unaltered to *Focn*	(Thatcher et al., 2009)
	ein2-5	ET signaling	Arabidopsis	increased to *Focn* and *Fol*	(Berrocal-Lobo and Molina, 2004)
	etr1-1	ET peception	Arabidopsis	reduced to *Forp*	(Pantelides et al., 2013)
	35S::ERF1	ET signaling	Arabidopsis	reduced to *Focn* and *Fol*	(Berrocal-Lobo and Molina, 2004)
	Never ripe	ET perception	tomato	reduced to *Fol*	(Lund et al., 1998; Francia et al., 2007)
	Never ripe, epinastic (epi1)	ET signaling	tomato	unaltered to *Forl*	(Kavroulakis et al., 2007)
ABA	*aba1-6, aba2-1*	ABA biosynthesis	Arabidopsis	reduced to *Focn*	(Anderson et al., 2004; Trusov et al., 2009)
	aba2	ABA biosynthesis	Arabidopsis	reduced to *Focn*	(Cole et al., 2014)
Auxin	*cyp79b2 cyp79b3, atr4/sur2, myb51/hig1, atr1, atr2d, pad3 35S:ATR1/MYB34, atr1d, 35S:ATR2,*	auxin biosynthesis	Arabidopsis	unaltered to *Focn*	(Kidd et al., 2011)
	axr1, axr2, axr3, sgt1b	auxin signaling	Arabidopsis	reduced to *Focn*	(Kidd et al., 2011)
	tir1	auxin perception	Arabidopsis	unaltered to *Focn*	(Kidd et al., 2011)
	arf1, arf2, arf1arf2	auxin signaling	Arabidopsis	reduced to *Focn*	(Lyons et al., 2015)

depends on growth and inoculation conditions, plant age and the *Fo* isolate used. It appears that at least in soil-grown plants, SA enhances immunity to *Fo* via NPR1- and EDS1-independent pathways.

Consistent with its defense promoting role in Arabidopsis, exogenous application of SA or synthetic SA analogs reduced *Fo* disease symptoms in a broad range of tested plants including tomato, common bean, date palm and Arabidopsis (Edgar et al., 2006; Mandal et al., 2009; Dihazi et al., 2011; Xue et al., 2014). Furthermore, stable overexpression of

Arabidopsis *NPR1* in tomato reduced disease symptoms as well as *Fo* f.sp. *lycopersici* (*Fol*) colonization of the stem (Lin et al., 2004). Similarly, preventing SA volatilization by silencing of a Salicylic Acid Methyltransferase reduced tomato susceptibility to *Fol*, however without significantly changing overall SA levels (Ament et al., 2010). How exactly Methyl-SA levels influence tomato defense to *Fol* remains to be addressed.

Despite a clear effect of SA on disease severity, global transcriptome profiling of Arabidopsis plants inoculated with *Focn* revealed relatively mild changes in the expression of known SA marker genes (Kidd et al., 2011; Zhu et al., 2013; Chen et al., 2014; Lyons et al., 2015). In fact, expression of *PR1* was even slightly down regulated both in roots and shoots of inoculated plants (Kidd et al., 2011). It is possible that activation of SA signaling occurs at rather late stages, which would be missed by the present studies that focus on the time points 1 to 6 days-post-inoculation (dpi). Alternatively, SA signaling could activate previously uncharacterized defense mechanisms, in line with the observed NPR1- and EDS1-independency, especially in roots that are still little explored in terms of plant immunity (De Coninck et al., 2015). A third possibility has been suggested by Cole et al.: SA signaling could serve to dampen activation of JA responses that are promoting Arabidopsis infection by *Fo* (see below) (Cole et al., 2014). Similar to Arabidopsis, transcriptome profiling of banana saplings infected with virulent and avirulent *Fo* f.sp. *cubense* (*Focb*) strains also failed to detect activation of typical SA marker genes, at least at the relatively early time points analyzed (Li et al., 2013). This led to the suggestion that in banana defense against *Fo* is mainly mediated via ET/JA signaling (Swarupa et al., 2014).

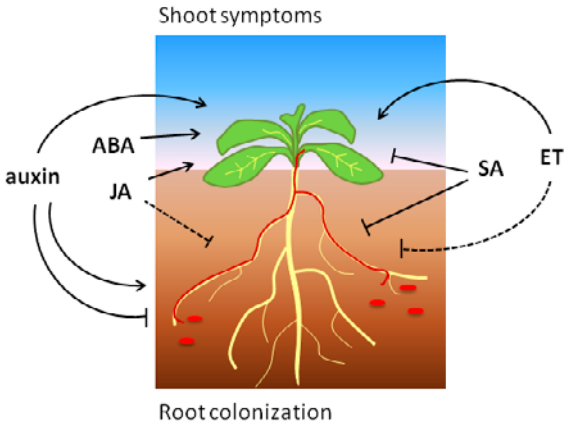

Figure 1. The effects of phytohormone signaling on Arabidopsis root colonization and shoot symptoms induced by *Focn* (shown in red). Arrows and blunt ends indicate promotion and reduction, respectively. The dashed line indicates a presumed positive role of ET-signaling on (root) defense.

Taken together, SA signaling positively regulates defense to *Fo* in most tested plant species, which is in line with a predominantly hemi-biotrophic lifestyle of this pathogen (Figure 1). However, the exact mechanisms by which SA reduces susceptibility to *Fo* are not understood and yet unknown SA targets possibly play a role during defense to this root-infecting pathogen.

JA signaling can promote either resistance or susceptibility in different host-*Fo* interactions

JA signaling generally mediates resistance to necrotrophic pathogens and insect herbivores. These two functions are exerted by two separate and often mutually antagonistic branches: the former is regulated by ERF transcription factors and is associated with ET signaling, whereas the latter requires MYC transcription factors and often involves ABA signaling (Pieterse et al., 2012).

In Arabidopsis JA biosynthesis is not critical for defense to *Focn* as a whole series of mutants with impaired JA accumulation behaved like WT plants (Table 2) (Thatcher et al., 2009). Similarly, *jar1* mutants that are defective in synthesis of the bioactive JA-Isoleucine conjugate showed WT-like or slightly increased susceptibility (Thatcher et al., 2009; Trusov et al., 2009). Considering these results it was unexpected that mutations in *CORONATINE INSENSITIVE1* (*COI1*), an essential component of JA perception, strongly increased resistance to *Focn* (Thatcher et al., 2009; Trusov et al., 2009). Importantly, loss-of-function mutations in additional regulators of JA signaling that are involved in pathogen defense, such as the transcriptional regulators MYC2, PFT1 and LBD20, also resulted in increased resistance to *Focn* (Anderson et al., 2004; Kidd et al., 2009; Thatcher et al., 2012b). These findings indicate that JA signaling-capacity of the host is critical for *Fo* pathogenesis in Arabidopsis.

Hijacking the host JA signaling machinery is a common strategy employed by many (hemi-)biotrophic pathogens and often involves suppression of SA-dependent defense responses (Pieterse et al., 2012; Kazan and Lyons, 2014). However, *COI1* promotes *Fo* infection in an SA-independent manner as *coi1 NahG* plants remain as resistant as *coi1* plants (Thatcher et al., 2009). This indicates that JA signaling supports *Fo* pathogenesis by mechanisms other than antagonizing SA responses. Grafting experiments demonstrated that expression of *COI1* in roots, but not in shoots, is required for *Fo* infection (Thatcher et al., 2009). Cole and co-workers observed reduced colonization of the vasculature in *coi1* plants pointing to a role of JA signaling at relatively early stages (Cole et al., 2014). Thatcher et al. detected similar levels of fungal biomass in WT and *coi1* shoots before the switch to necrotrophic growth, and hence concluded that *COI1* is predominantly required for triggering plant decay (Thatcher et al., 2009).

These differences might be explained by the inoculation methods used: uprooting of plants before inoculation could have created additional entry sites leading to stronger vascular colonization (as observed in (Thatcher et al., 2009)). Nevertheless, the studies indicate that COI1 influences the interaction with *Fo* at several stages. Moreover, the finding that even strongly colonized *coi1* plants remain essentially symptomless reveals an uncoupling of colonization and plant disease symptoms (Thatcher et al., 2009).

Despite its strong effects on the interaction with *Fo*, it is not well understood how the host JA signaling machinery is re-wired by the pathogen to promote disease. Cole and co-workers found that two Arabidopsis-infecting strains, *Focn* and f.sp. *matthioli* (*Fomt*) produce JA-isoleucin and JA-leucin conjugates in culture filtrates that induce senescence-like symptoms on Arabidopsis leaves in a COI1-dependent manner (Cole et al., 2014). However it is not yet known if these fungal derived hormones are also generated during infection, and to what extent they contribute to virulence. Alternatively, *Fo* effectors could play a role in JA signaling manipulation as has been suggested for the effector SIX4 (Thatcher et al., 2012a). It will be interesting to explore which JA signaling regulators are targeted by *Fo* and how their activity is modulated.

The dependency on host JA signaling for successful colonization however, is not conserved among all *Fo* ff.spp. For instance, *Forp* colonizes WT and *coi1* Arabidopsis plants to a similar extent and this isolate does not produce bioactive JA-conjugates *in vitro*. Similarly, *Fol* seems to infect tomato without JA-signaling manipulation as this f.sp. does not produce JAs and is not dependent on the tomato *COI1* homologue to cause disease (Cole et al., 2014). Thus, different *Fo* isolates have developed distinct infection strategies that either strongly rely on host JA signaling manipulation or involve alternative virulence mechanisms that are JA-independent.

In addition, several lines of evidence point to a role of JA in promoting resistance rather than susceptibility in plant species other than Arabidopsis. Tomato *def1* mutants that are defective in JA synthesis show enhanced susceptibility to *Fol*, which can be suppressed by exogenous JA treatment (Thaler et al., 2004). Similarly, *def1* tomato plants were more susceptible to root rot caused by *Fo* f.sp. *radicis-lycopersici* (*Forl*) (Kavroulakis et al., 2007). Consistently, Sun and co-workers found that spraying of banana plants with Methyl-JA reduced disease incidence and severity caused by *Focb* Tropical Race 4 (Sun et al., 2013). In addition, resistant cultivars of strawberry and watermelon showed strong induction of the JA biosynthesis enzyme AOC upon inoculation with *Fo* f.sp. *fragariae* (Lu et al., 2011; Fang et al., 2013). A positive role of JA for defense activation was also found in date palms inoculated with *Fo* f.sp. *albenidis* (Jaiti et al., 2009).

In summary, JA signaling promotes defense to *Fo* in different plant species, but can also

be hijacked to induce pathogenicity in at least Arabidopsis (Figure 1). Further research will be necessary to reveal via which mechanisms JA contributes to disease reduction or induction, and which function is predominant among different plant species.

Dual role of ET in activation of both defense responses and disease symptoms

Generally, ET together with JA mediates the resistance response to necrotrophic pathogens. However, ET can also positively influence defense responses to hemi-biotrophs and induced systemic resistance, which is triggered by beneficial root-associated microbes (Pieterse et al., 2014; Broekgaarden et al., 2015).

Pre-treatment of Arabidopsis seedlings with either MeJA or the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) resulted in enhanced disease symptoms upon *Focn* inoculation, indicating that both these hormones promote disease development (Trusov et al., 2009). Accordingly, the ET-insensitive Arabidopsis *ein2-1* and *etr1-1* mutants showed a reduction of disease symptoms compared to WT Col plants when inoculated with *Focn* or *Forp*, respectively (Table 2) (Trusov et al., 2009; Pantelides et al., 2013). It is worth mentioning that enhanced disease upon MeJA treatment, as well as reduced disease in ET-insensitive plants was not observed in similar studies, indicating that these effects are either weak or depend on yet unknown factors (Edgar et al., 2006; Thatcher et al., 2009). Moreover, a different *ein2* allele (*ein2-5*) even showed markedly enhanced susceptibility to *Focn* under sterile conditions (Berrocal-Lobo and Molina, 2004). These findings point to an age- and/or condition-dependent role of ET in Arabidopsis interaction with *Fo*.

Global transcriptome profiling in Arabidopsis and banana plants inoculated with virulent *Fo* strains revealed a massive induction of ET/JA-responsive genes such as *Plant Defensins* (*PDFs*) and *Pathogenesis-Related* (*PR*) genes as well as genes encoding ethylene biosynthesis enzymes (Kidd et al., 2011; Li et al., 2013; Zhu et al., 2013; Lyons et al., 2015; McGrath et al., 2005). Furthermore, the transcriptome profiles indicated that initial activation of ET-dependent genes precedes the activation of JA, SA and ABA signaling (Li et al., 2013; Zhu et al., 2013). Altogether, these findings suggest a model in which initial ET/JA-associated defenses are mounted in response to *Fo* infection, but these are typically insufficient to stop the pathogen. At later stages however, ET signaling can rather enhance disease symptoms and possibly also pathogen proliferation. This hypothesis is further supported by the observation that Arabidopsis plants overexpressing certain *ERF* transcription factors, thereby constitutively activating ET/JA-dependent defense responses, become less susceptible to *Focn* (Berrocal-Lobo and Molina, 2004; McGrath et al., 2005). However, whether ET signaling is actively suppressed and/or at later stages co-opted by *Fo* to promote pathogenesis remains to

be addressed.

The exact role of ET in other plant species is relatively little understood. The tomato *Never ripe* (*Nr*) mutant is impaired in ethylene perception and shows reduced disease symptoms upon *Fol* inoculation (Lund et al., 1998; Francia et al., 2007). Interestingly, previous work revealed a role for ET in mediating xylem occlusion through formation of gels in castor bean (Vandermolen et al., 1983). Xylem occlusion is thought to limit pathogen spread, but also to contribute to the typical wilting symptoms (Yadeta and Thomma, 2013). Thus it is an interesting question whether *Nr* tomato plants allow systemic fungal spread and how this would correspond to the observed reduction in disease symptoms. Resistance to *Forl*, a pathogen which adopts a necrotroph-like lifestyle, was largely unaffected in two tested ET-insensitive tomato lines, *Nr* and *epinastic* (*epi*) (Kavroulakis et al., 2007). However, protection mediated by an endophytic *Fusarium solani* strain was greatly reduced in *Nr* and *epi* tomato plants and hence required intact ET signaling (Kavroulakis et al., 2007).

In conclusion, the present studies underline a multifaceted role of ET signaling that strongly depends on the interaction stage, the host plant and environmental conditions (Figure 1).

ABA promotes shoot disease symptoms but not root colonization in Arabidopsis

Besides its well described role in development and abiotic stress responses, ABA has been increasingly recognized as a critical regulator of biotic interactions. ABA can either positively or negatively influence resistance largely depending on the encountered pathogen (Ton et al., 2009; Robert-Seilaniantz et al., 2011).

Similarly to Methyl-JA and ACC, exogenous treatment with ABA increased Arabidopsis susceptibility to *Focn* (Trusov et al., 2009). Consistently, Arabidopsis mutants in which either ABA biosynthesis or signaling is disrupted showed fewer symptoms (Table 2) (Anderson et al., 2004; Trusov et al., 2009). The reduced susceptibility of ABA mutants was associated with hyper-activation of ET/JA-dependent defense genes, likely due to antagonistic interactions between ABA and ET signaling (Anderson et al., 2004). ABA could also antagonize SA-dependent responses (Yasuda et al., 2008), but currently it is unknown if the interaction with other hormones explains reduced *Fo* symptoms in ABA-deficient mutants.

Interestingly, *Fo* successfully colonized the roots of ABA-deficient mutants to a similar extent as those from WT plants (Cole et al., 2014). This would point to a role of ABA during the switch to the necrotrophic phase. However, transcriptome profiling

revealed activation of numerous ABA responsive genes in the roots of *Fo*-inoculated plants (Lyons et al., 2015). Previous studies indicated that ABA mediates root-to-shoot defense signaling in plants (Balmer et al., 2013). This raises the possibility that *Fo* co-opts systemic ABA signaling to manipulate root-shoot signaling. Moreover, it has been shown that, for example during defense against herbivorous insects, ABA signaling can serve to activate or enhance the MYC2-regulated branch of JA signaling (Kazan and Manners, 2013; Vos et al., 2013). However, if and how exactly *Fo* manipulates ABA signaling, is currently unknown.

Auxins affects both root colonization and shoot symptom development

Auxins are major regulators of plant growth and development, but have also profound effects on interactions with both pathogenic and mutualistic microbes (Robert-Seilaniantz et al., 2011; Zamioudis et al., 2013).

Exogenous application of auxin or auxin biosynthesis inhibitors did not affect disease development in *Focn*-inoculated Arabidopsis (Kidd et al., 2011). Similarly, mutants with either reduced or increased auxin levels behaved like WT plants. In contrast, *Focn*-inoculated auxin-signaling mutants showed markedly reduced symptoms relative to WT plants (Table 2). Additionally, alteration of polar auxin transport, either by chemical inhibitors or in mutants, resulted in increased resistance to *Focn* (Kidd et al., 2011). These data indicate that local changes of auxin levels and/or distribution are important for disease susceptibility. Indeed, histological visualization of *DR5* expression, a well-known auxin reporter gene, revealed activation of auxin signaling at root tips and lateral root initials, two preferred *Fo* entry sites in Arabidopsis (Czymmek et al., 2007; Kidd et al., 2011; Diener, 2012). Additionally, Diener revealed that fewer root tips are colonized in plants mutated in the auxin efflux carrier *PIN2/EIR1* (Diener, 2012). In contrast, *tir3* mutants which are defective in polar auxin transport show 2-3 fold higher *Fo* biomass in roots, but disease symptoms of the shoot remained strongly reduced (Kidd et al., 2011; Diener, 2012). These findings suggest that auxin signaling and transport affect several stages of the *Fo*-Arabidopsis interaction from initial root tip colonization to disease symptom expression in the shoot. However, the mechanisms by which auxin promotes colonization and symptom development are still enigmatic. Previous studies describe an antagonistic relationship between auxin and SA signaling, however the *Fo* disease phenotypes of auxin signaling mutants were not SA-dependent (Kidd et al., 2011).

Auxin accumulation, transport and signaling are modulated by numerous different symbiotic and pathogenic organisms including bacteria, fungi, nematodes and even parasitic plants during their interaction with roots (Grunewald et al., 2009; Kazan and Manners, 2009; Zamioudis and Pieterse, 2012). This suggests that manipulation of the

host auxin signaling pathway represents a common strategy employed by diverse root colonizers resulting in either detrimental or beneficial effects for plants.

Are phytohormones determinants of *Fo* lifestyle?

Interestingly, changes in the phytohormone network can uncouple colonization by *Fo* from plant disease development. For instance, specific mutants with impaired JA, ABA and auxin signaling still allow extensive root (and sometimes shoot) colonization but have greatly reduced disease symptoms (Thatcher et al., 2009; Diener, 2012; Cole et al., 2014). Similarly, resistant tomato plants that are completely free of symptoms can have their shoots and stems extensively colonized by *Fol* (Mes et al., 1999). Furthermore, a *Fol* knockout strain lacking the Six6 effector triggered vascular browning in a susceptible tomato cultivar, indicative of successful xylem colonization, but exerted almost no negative effects on plant growth and development (Gawehns et al., 2014). Altogether, these findings suggest that manipulation of plant hormone signaling rather than colonization triggers disease symptom development.

How does *Fo* manipulate the plant hormone network? One mechanism could be the production by the fungus of hormone-like secondary metabolites, including JAs, auxins, gibberellic acids and ethylene (Hasan, 2002; Cole et al., 2014; Bitas et al., 2015). *Fo* also secretes numerous small proteins during plant infection, which might be another means to manipulate the host. For several of these proteins a virulence-promoting function has been shown designating them as effectors *sensu strictu* (Takken and Rep, 2010; de Sain and Rep, 2015). Among these, SIX4 was found to enhance JA signaling during infection of Arabidopsis (Thatcher et al., 2012a). Infection of tomato plants with *Fol* knockout strains lacking specific effectors revealed common and unique effects on the xylem proteome composition raising the possibility that each effector targets a distinct hormone signaling pathway (Gawehns et al., 2015). However, for the vast majority of *Fo* effectors their working mechanism remains unknown. It will be interesting to explore if plant hormone-synthesis or -signaling represents a recurrent virulence target of *Fo* strains on various hosts. Furthermore it is tempting to speculate that at least a subset of the proteinacious effectors mediate immune suppression enabling (endophytic) colonization during the biotrophic phase of infection, whereas secondary metabolites with hormonal- or toxic-activity trigger plant damage/death during necrotrophic growth. A growing body of evidence suggests that the majority of *Fo* strains survive in soil, in the rhizosphere or within plant tissues without causing disease symptoms, and some strains even confer extensive beneficial effects (Alabouvette et al., 2009; Edel-Hermann et al., 2015; Imazaki and Kadota, 2015). The existence of such a widespread and intimate co-habitation points to a finely balanced interaction between plant and fungus. The finding that also pathogenic strains can reside inside plant tissues without damaging the

host indicate that one *Fo* isolate can employ diverse interaction/colonization strategies whose outcome possibly depends on the "compatibility" of a putative host plant. Thus, the 'infection tools' of a *Fo* strain, likely comprised of a combination of effectors, enzymes and secondary metabolites, determine the outcome of an interaction: either endophytic with potential beneficial effects for the host or pathogenic with various levels of disease and in extreme cases plant death. This idea is supported by the observation that transfer of one specific *Fol* chromosome, which contains most of its effector genes plus a secondary metabolite cluster, conferred pathogenicity to an endophytic strain (Ma et al., 2010).

Clearly more research in the areas of genomics and effector biology is required to understand how *Fo* manages to trick the hormonal network of its hosts, and how these interactions can have opposite outcomes ranging from pathogenesis to mutualism.

Conclusions and outlook

Phytohormones determine colonization and disease symptom development during interactions with pathogenic *Fo* strains. However, their roles vary depending on the host plant and the fungal strain involved, suggesting that the manipulation of the host hormonal network differs between individual *Fo* strains. Genetic interference with hormone regulators mostly reduced disease symptoms, as seen for JA, ABA and auxin, indicating that the ability to hijack plant hormone pathways is a requirement for pathogenesis. This scenario implies a strong adaptation to a particular host plant, potentially leading to the narrow host range observed in the *Fo* species complex. How exactly the manipulation of phytohormone signaling differs between Fo strains - and if this is indeed the key difference between pathogenic and endophytic interactions - remains an intriguing question for future research. Comparison of the respective effector repertoires as well as a better understanding of their mode of action will help answering these questions and may furthermore reveal novel approaches for plant protection, either by breeding or by optimizing *Fo* strains for biocontrol.

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Chapter 3

Involvement of SA, ET and JA signaling pathways in susceptibility of tomato for *Fusarium oxysporum*

This chapter has been provisionally accepted:
Di,X., Gomila, J., Takken, F. L. W. (2017) Involvement of salicylic acid, ethylene and jasmonate acid signaling pathways in susceptibility of tomato for *Fusarium oxysporum.* Molecular Plant Pathology

Abstract

Phytohormones such as salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) play key roles in plant defense following pathogen attack. Involvement of these hormones in plant susceptibility following *Fusarium oxysporum* (*Fo*) infection has mostly been studied in *Arabidopsis thaliana*. However, *Fo* causes vascular wilt disease in a broad range of crops, including tomato (*Solanum lycopersicum*). Surprisingly little is known about the involvement of these phytohormones in tomato toward *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) susceptibility. Here we investigate their involvement by analyzing the expression of ET and SA marker genes, following *Fol* infection and using bioassays on tomato mutants affected in either hormone production or -perception. *Fol* inoculation triggered expression of SA and ET marker genes showing activation of these pathways. *NahG* tomato in which SA is degraded, was hyper-susceptible to *Fol* infection and exerted stronger disease symptoms than wild-type. In contrast *ACD* and *Never ripe* (*Nr*) mutants in which respectively ET biosynthesis or -perception is impaired, showed decreased disease symptom development and a reduced fungal colonization upon infection. Susceptibility of the *def1* tomato mutant, which is deficient in JA biosynthesis, was unaltered, while a prosystemin over-expressing line, showed slightly more disease symptoms concomitantly with increased fungal colonization. The latter is surprising as prosystemin indirectly regulates JA biosynthesis. Our results show that SA is a negative and ET a positive regulator of susceptibility. The SA and ET signaling pathways appear to act synergistically as an intact ET pathway is required for induction of the SA reporter gene and *vice versa*.

Key words: *Fusarium oxysporum*, SA, ET, JA, tomato, susceptibility

Introduction

The root-infecting fungal pathogen *Fusarium oxysporum* (*Fo*) causes vascular wilt disease in over 100 different plant species, including banana, cotton, palm, Arabidopsis and tomato (Michielse and Rep, 2009). *Fo* represents a species complex comprising many individual pathogenic strains, each capable to infect one or a few host species only. Based on host specifity, strains have been grouped into *formae speciales*. Infection by *Fo* starts upon its attachment to the plant root surface. Fungal hyphae enter roots through wounds or cracks at the root tip or at sites of lateral root formation. Ultimately the fungus reaches the xylem vessels and proliferates there. Subsequently, disease ensues (Rep et al., 2002, di Pietro et al., 2003, Berrocal-Lobo and Molina, 2008). In attempting to arrest the pathogen when it spreads through the vascular tissues, the plant blocks its infected vessels thereby compromising their ability to transport water and nutrients. Vascular browning, stunting, progressive wilting and eventually plant death are typical disease symptoms in infected plants (di Pietro et al., 2003, Agrios, 2005).

In general, plant defense responses against pathogens are controlled by complex signalling routes often involving the classical defense-phytohormones salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) (Robert-Seilaniantz et al., 2011). Usually, SA signaling triggers resistance against biotrophic and hemibiotrophic pathogens, whereas a combination of JA and ET signaling activates resistance against necrotrophs (Glazebrook, 2005).

Due to the extensive availability of genetic and genomic resources most studies on phytohormone involvement in defense against *Fo* have been performed in Arabidopsis. (Edgar et al., 2006). Arabidopsis is susceptible to *Fo forma specialis* (f.sp.) *conglutinans* (*Focn*). Arabidopsis lines that express the salicylate hydroxylase transgene (*NahG*), or that carry the SA *induction-deficient 2* (*sid2*) mutant, are impaired in SA accumulation. Both lines exert increased susceptibility to *Fo* showing involvement of SA in disease susceptibility (Berrocal-Lobo and Molina, 2004, Diener and Ausubel, 2005).

Pre-treatment of Arabidopsis seedlings with either Methyl JA (MeJA) or the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) led to enhanced disease symptom development upon *Fo* inoculation, indicating that both ET and JA are involved in disease susceptibility (Trusov et al., 2009). The ethylene-insensitive Arabidopsis mutants *ein2* and *etr1-1* showed a reduction of disease symptoms compared to Col-0 plants when inoculated with *Focn* (Trusov et al., 2009, Pantelides et al., 2013). In contrast, various JA biosynthesis mutants such as *jasmonate resistant 1* (*jar1-1*) and *allene oxide synthase* (*aos*) did not exhibit increased susceptibility to *Fo* (Thatcher et al., 2009, Trusov et

al., 2009). Surprisingly, a point mutation in *CORONATINE INSENSITIVE* 1 (*COI1*), an essential component of JA perception, strongly reduced disease symptom development following *Fo* infection (Thatcher et al., 2009, Trusov et al., 2009). Additionally, disruption of *MYC2*, *PFT1* and *LBD20*, transcriptional regulators of JA signaling, also resulted in an increased resistance to *Fo* (Anderson et al., 2004, Kidd et al., 2009, Thatcher et al., 2012). Taken together, ET and JA are positive regulator of susceptibility in Arabidopsis.

The role of phytohormones in determining host colonization and disease symptom development is known to vary for different *formae speciales* of *Fo* and their respective hosts (Di et al., 2016). To obtain a better insight in these processes it is therefore crucial to investigate the role of phytohormones in defense responses to *Fo* in plant species other than Arabidopsis. Tomato (*Solanum lycopersicum*), a major and important vegetable crop (Panthee and Chen, 2010), is susceptible to *Fo* f.sp. *lycopersici* (*Fol*) resulting in significant yield losses each year (McGovern, 2015). The interaction between tomato and *Fol* is well studied (Takken and Rep, 2010). Like other *formae speciales* of *Fo Fol* colonizes the vasculature and infected plants exhibit vascular browning, leaf epinasty, stunting, progressive wilting and eventually death (di Pietro et al., 2003). During colonization the fungus secretes virulence factors called effector proteins (Houterman et al., 2007). Deletion of specific effectors, such as Avr2, compromises virulence of the fungus making it less pathogenic than wild-type *Fol* (Houterman et al., 2007, Houterman et al., 2009).

For tomato a great collection of lines is available that are compromised either in hormone perception, -metabolism or -signaling. In our study, mutants affected in the biosynthesis and signaling pathway of specific defense-related hormones were analyzed for their susceptibility to *Fol*. The lines used included; transgenic *NahG* plants that express the bacterial enzyme salicylate hydroxylase, which converts SA into biologically inactive catechol, making the plants deficient in SA accumulation (Brading et al., 2000). *Never ripe*, a dominant ET-insensitive mutant, carrying a single base substitution in the region encoding the N-terminus of ETR3, which is a homolog of the Arabidopsis ETR1 receptor (Wilkinson et al., 1995). The transgenic tomato line *ACD*, which expresses the *ACCD* (1-amino-cyclopropane-1-carboxylic acid deaminase) gene; encoding the ACCd enzyme that catalyzes the degradation of ACC. JA-deficient mutant *defenseless-1* (*def1*), which has a defect in the jasmonate pathway between 13-HPOTE and 12-oxo-PDA. This mutant fails to form JA and to systemically accumulate proteinase inhibitors in response to systemin and oligosaccharide elicitors (chitosan and polygalacturonide) (Li et al., 2002). In addition, a *35S::prosystemin* transgenic line overexpresses prosystemin was included. Prosystemin is a positive regulator of JA signaling, and hence the plants constitutively accumulate high levels of proteinase inhibitor proteins (Howe and Ryan, 1999).

Here, we report on our inoculation assays of the various tomato lines affected in SA, ET and JA signaling with wild-type *Fol* and the *FolΔAvr2* mutant. In contrast to JA signaling, which appears to be involved mainly in the ability of the fungus to colonize the plant, SA and ET have major and opposing roles in disease susceptibility and development. The SA and ET signaling pathways appear to act synergistically as an intact ET pathway is required for induction of the SA reporter gene and *vice versa*. A model for the role of SA, ET and JA signaling in tomato toward susceptibility against *Fol* is proposed and compared to that in Arabidopsis.

Results

NahG tomato plants show enhanced disease symptom development upon *Fol* infection

To assess a potential role of SA in modulating susceptibility against *Fol*, three-week-old wild-type tomato plants (cultivar Moneymaker) and transgenic *NahG* plants impaired in SA accumulation, were inoculated with either water (mock) or wild-type *Fol*, notably race 2 isolate *Fol007*. Additionally, to allow assessment of hyper-susceptibility, a *Fol007 Avr2* knockout strain (*FolΔAvr2*) was included. This mutant is compromised in virulence and causes less disease symptoms on susceptible plants (Houterman et al., 2009). As shown in Figure 1A, *NahG* plants inoculated with *Fol007* exhibited more severe disease symptoms than wild-type plants. These symptoms include extensive wilting and a more severe stunting three weeks after inoculation. Consistent with this, the fresh weight of *Fol007*-infected *NahG* tomato plants was significantly lower than that of corresponding wild-type plants. Moreover, all vascular bundles of infected *NahG* plants had turned brown, and plants were either dead or very small and wilted. On a scale from 0 to 4 (Gawehns et al., 2014) the infected *NahG* plants scored the maximal disease index (Figure 1B and C). As expected, *FolΔAvr2*-inoculated plants developed less severe disease symptoms (Figure 1A). Similar to *Fol007*, *FolΔAvr2* infected *NahG* plants showed a significant reduction in fresh weigh and a higher disease index as compared to wild-type plants (Figure 1B and C).

To investigate whether the augmented disease symptom development in *NahG* plants correlated with increased host colonization, a fungal recovery assay was performed. Thereto, sections were taken from *Fol*-inoculated wild-type and *NahG* plants at different heights of the stem, notably at the position of 1) the cotyledon, 2) the second node and 3) the fourth node, and upon sterilization placed on CDA plates and incubated for 5 days at 25°C. As shown in Figure 1D, in 30% of the cases the fungus was observed in stem sections collected at the fourth node of *Fol007*-inoculated wild-type plants, whereas in 70% of the cases of that in *Fol007*-inoculated *NahG* plants. Typically, colonization of

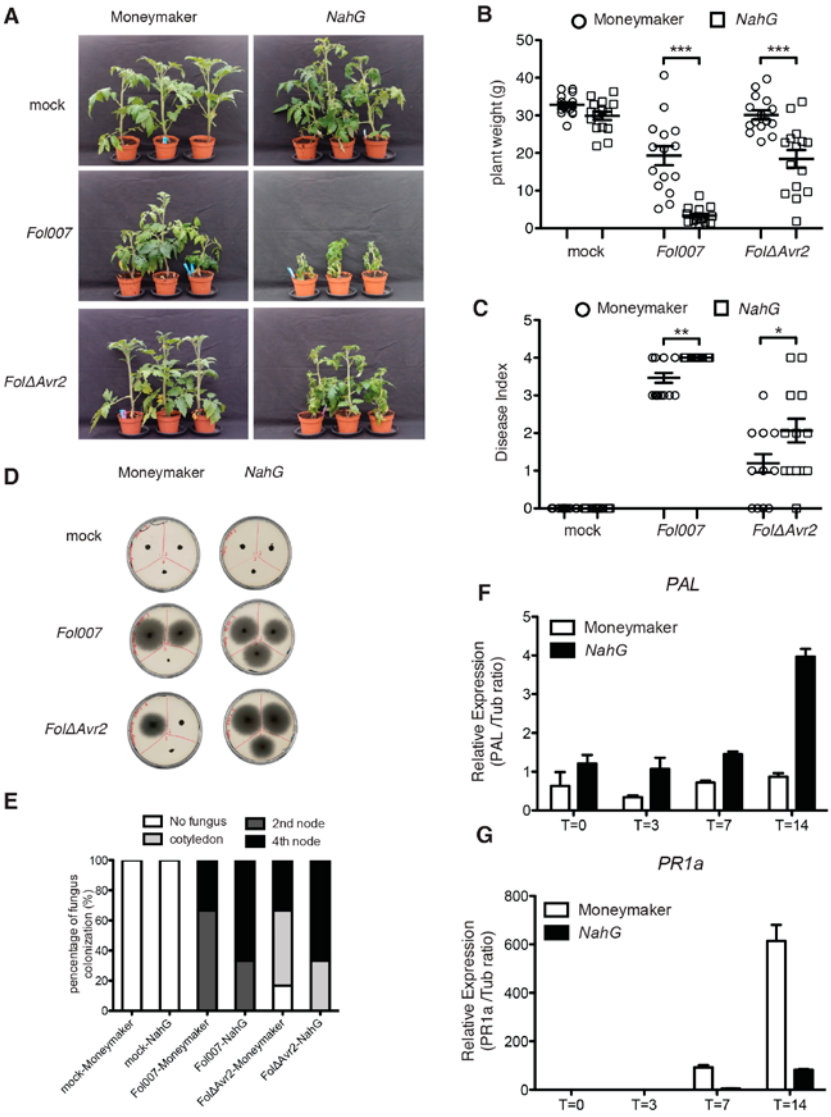

Figure 1. Impaired SA signaling enhances *Fol* disease symptom development in tomato. (A). Three-week-old wild-type Moneymaker and *NahG* tomato plants inoculated with either water (mock), *Fol007* or *FolΔAvr2* at 21 days post-infection (dpi). (B) Disease development was scored by measuring fresh plant weight and (C) determining the disease index (ranging from 0-4) of 20 plants/genotype. Circles and squares indicate respectively Moneymaker and *NahG* plants. Plant weight and disease index were subjected to a pairwise comparison with a Student's *t*-test (*P<0.05; ** P<0.01; ***P<0.001). The bioassay was repeated three times with similar results. (D). Stem sections from cotyledon (top-left), 2nd node (top-right) and 4th node (bottom-center) of individual *Fol*-inoculated plants (n=6) after incubation for five days on CDA plates. (E). Percentage of infected slices showing fungal outgrowth. Fungal progression in the stem was expressed as infected percentage of all stem pieces. (F) Transcription patterns of *PAL* and (G) *PR1a* in *Fol007*-inoculated wild-type Moneymaker and *NahG* at 0, 3, 7 and 14 dpi. Gene expression levels relative to the internal control tubulin genes were quantified by qPCR.

wild-type plants was much reduced from stem sections of *FolΔAvr2*-inoculated wild-type plants, while *FolΔAvr2* was found to efficiently colonize *NahG* plants as fungal outgrowth was often observed up to the fourth node (Figure 1E). Together these data suggest that *NahG* plants are hyper-susceptible to *Fusarium* infection and that the increased symptoms correlate with increased fungal colonization of the transgenic plants.

SA is biosynthesized in the phenylpropanoid pathway through the activity of phenylalanine ammonia-lyase (PAL) (Lee et al., 1995). To assess expression of the *PAL* gene during infection, an RT-qPCR analysis was carried out on hypocotyls. Samples were taken at 0, 3, 7 and 14 days after inoculation of wild-type and *NahG* plants, and expression levels were measured and normalized to tubulin. We found that at 14dpi transcript levels of *PAL* were up-regulated in *NahG* plants compared to wild-type plants (Figure 1F).

Very often *PR1a* expression is used as a reporter for SA-dependent defense signaling (Kunkel and Brooks, 2002). To assess whether *PR1a* expression is altered during *Fol* infection, its expression at 0, 3, 7 and 14 days after inoculation was measured. Transcript levels of *PR1a* were strongly induced in wild-type plants upon infection, suggesting that SA signaling is activated during *Fol* infection (Figure 1G). Compared to wild-type plants the expression of *PR1a* in infected *NahG* plants was very much reduced. This correlates with the loss of SA accumulation in *NahG* plants, confirming the proposed phenotype of the transgenic line. Overall, these data suggest that impaired SA signaling enhances *Fol* susceptibility and disease symptom development in tomato.

ET enhances susceptibility to *Fol* in tomato plants

Pre-treatment of Arabidopsis seedlings with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) leads to enhanced disease symptom development upon *Focn* inoculation, indicating that ET is involved in disease susceptibility (Trusov et al., 2009). To investigate the role of ET in disease symptom development in tomato, transgenic plants impaired in ET-biosynthesis were analyzed for their susceptibility to *Fol* infection. In the transgenic line *ACD*, constitutively expressing a bacterial 1-amino-cyclopropane-1-carboxylic acid deaminase gene, ET production is reduced 90% compared to wild–type (Klee et al., 1991). Since the transgene is present in cultivar UC82B, this cultivar was used as wild-type control. Whereas UC82B showed severe wilting and stunting following *Fol* infection, most *ACD* plants showed only mild disease symptoms (Figure 2A). Plant weight of infected *ACD* plants was significantly higher than that of infected wild-type plants (Figure 2B). In addition, the disease index in *ACD* plants was also significantly attenuated as compared to the wild-type plants (Figure 2C). A similar reduction in

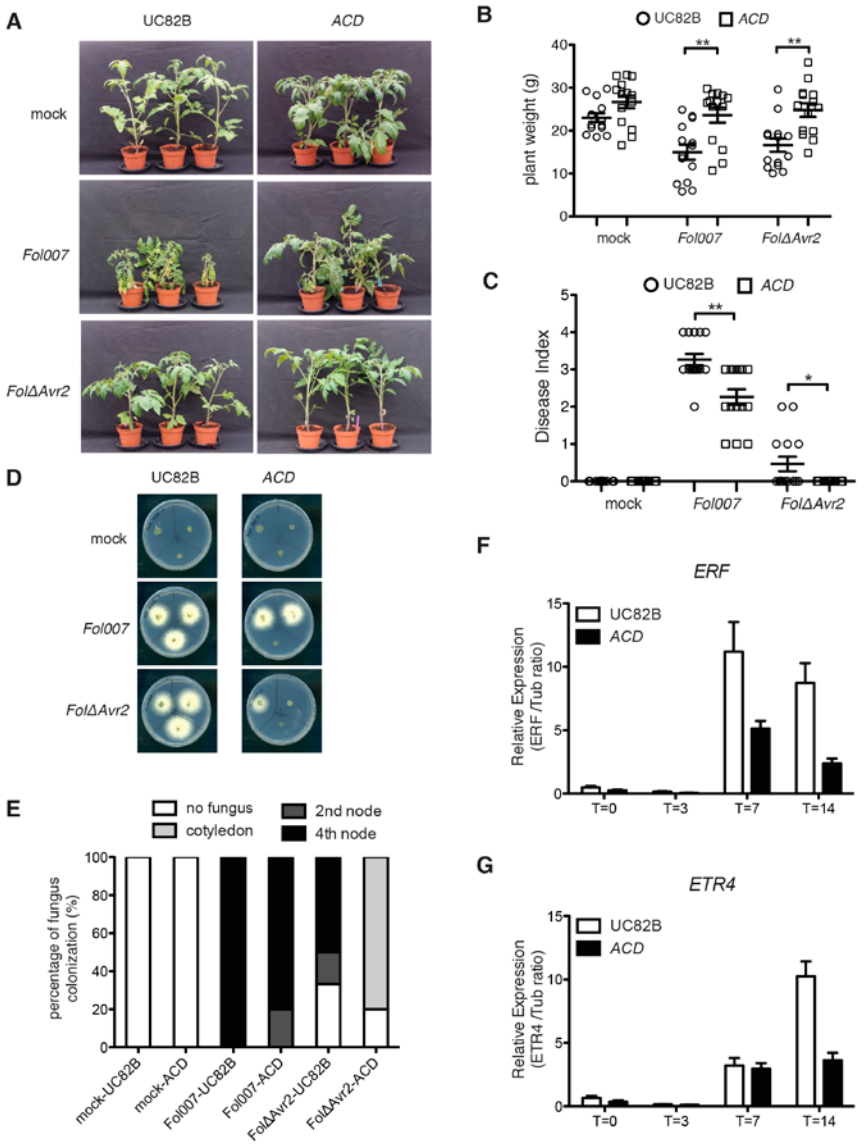

Figure 2. Impaired ET biosynthesis and production in tomato reduces disease susceptibility to *Fol*. (A). Three-week-old wild-type UC82B and *ACD* tomato plants inoculated with water (mock), *Fol007* or *FolΔAvr2* at 21 dpi. Disease development was scored by measuring (B) fresh weight and (C) disease index (ranging from 0-4). Circles and squares indicate UC82B and *ACD* plants (n = 20), respectively. Plant weight and disease index were subjected to a pairwise comparison using a Student's *t*-test (*P<0.05; ** P<0.01; ***P<0.001). The bioassay was repeated three times with similar results. (D). Stem sections from cotyledon (top-left), 2nd node (top-right) and 4th node (bottom-center) of individual plants (n=6) after five days of incubation on CDA plates. (E). Colonization is expressed as the percentage of infected slices of all stem pieces (n=6). (F). Transcription patterns of ET-regulated marker genes *ERF* and *ETR4* in *Fol007*-inoculated UC82B and *ACD* at 0, 3, 7 and 14 dpi. Gene expression levels relative to the internal control tubulin genes were quantified by qPCR.

symptom development was also observed in *ACD* lines inoculated with *FolΔAvr2*. To monitor fungal colonization stem sections were taken (see above) and incubated on CDA plates. The fungal recovery assay showed that *Fol007* grew out from most stem sections of both wild-type and the *ACD* line, whereas much less fungal growth was observed in *FolΔAvr2*-inoculated *ACD* plants. A typical example of a plate assay is shown in Figure 2D and the data from fungal recovery assay is summarized in Figure 2E. *FolΔAvr2* was found to efficiently colonize wild-type plants as fungal outgrowth was often observed up to the fourth node. Colonization of *ACD* plants was much reduced: in 80% of the cases the fungus was only observed in stem sections collected at the cotyledons. These data indicate that the *ACD* line exerts a reduced susceptibility towards Fol infection concomitantly with a reduction in symptom development.

It has been reported before that the ET receptor gene *ETR* and ET responsive factor *ERF* are induced by *Fo* infection (Berrocal-Lobo and Molina, 2008, Pantelides et al., 2013). As shown in Figure 2F and 2G, transcription of *ETR4* and *ERF* was highly induced in wild-type plants following *Fol* inoculation. Compared to wild-type, *Fol* infected *ACD* plants showed reduced expression of the tested genes. Apparently, the lack of ET accumulation in the *ACD* line results in a reduction of *ETR4* and *ERF* expression suggesting that the ET mediated signaling pathway is activated during *Fol* infection.

To distinguish whether the ET synthesis contributes to increased susceptibility by affecting the virulence of the fungus or the responses of the plant, the involvement of ET perception by the host was investigated. To study whether ET perception is required for disease symptom development following *Fol* infection, bioassays were performed with wild-type tomato cultivar Pearson and the ET insensitive Pearson mutant *Never-ripe* (*Nr*). Upon inoculation of wild-type Pearson with *Fol007*, the older leaves of infected plants became chlorotic and the plants showed mild wilting symptoms (Figure 3A). Upon inoculation with *FolΔAvr2*, wild-type plants showed hardly any symptom (Figure 3A). Notably, no obvious disease symptoms were observed in inoculated *Nr* plants. Although the fresh weight of *Nr* plants was identical to that of Pearson plants after *Fol* infection (Figure 3B), the *Nr* plants exhibited a significantly lower disease index than Pearson, as fewer brown vessels were observed in the stems (Figure 3C). Fungal recovery assays revealed that *FolΔAvr2* either completely failed to colonize *Nr* plants, or in the rare cases it could, it only reached the basal part of the stem that forms the hypocotyl (Figure 3D and E). Taken together the data suggest that the inability to synthesize ethylene as well as the perception of the hormone are essential for both disease development and the ability of the fungus to colonize the plant.

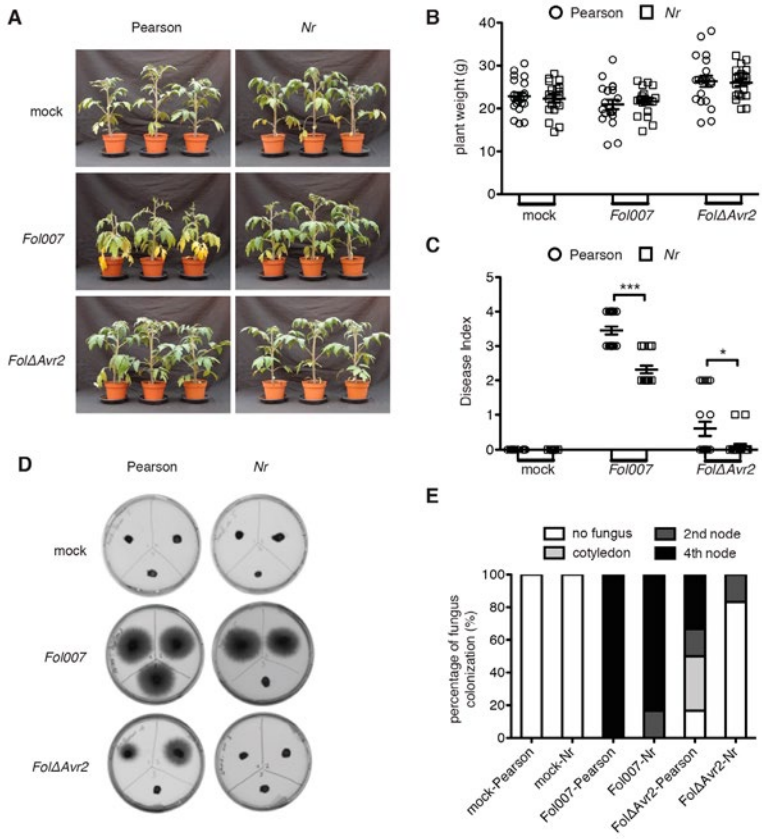

Figure 3. ET perception is required for *Fol* disease symptom development in tomato. (A). Three-week-old wild-type and *Nr* Pearson tomato plants inoculated with water (mock), *Fol007* or *FolΔAvr2* at 21 dpi. Disease symptoms were scored by measuring (B) fresh weight and (C) disease index (ranging from 0-4). Circle and square indicate Pearson plant and *Nr* plant (n = 20), respectively. Plant weight and disease index were subjected to a pairwise comparison using a Student's *t*-test (*P<0.05; ** P<0.01; ***P<0.001). The bioassay was repeated three times with similar results. (D). Stem sections from cotyledon (top-left), 2nd node (top-right) and 4th node (bottom-center) of individual plants (n=6) after five days of incubation on CDA plates. (E). Colonization is expressed as the percentage of infected slices of all stem pieces (n=6).

JA plays a minor role in the ability of the fungus to colonize tomato

In Arabidopsis, various JA biosynthesis mutants such as *jasmonate resistant 1* (*jar1-1*) and *allene oxide synthase* (*aos*) do not exhibit increased susceptibility to *Focn* (Thatcher et al., 2009, Trusov et al., 2009). To investigate the role of JA biosynthesis in susceptibility of tomato to *Fol*, we selected the *def1* mutant. This line has a defect in its octadecanoid biosynthesis pathway, which provides the precursors for JA synthesis, making the plant hyper-susceptible to herbivores due to its impaired accumulation of proteinase inhibitors (PIs) I and II in response to wounding (Lightner et al., 1993). Besides *def1* also *35S::prosystemin* plants were analyzed. In these plants the prosystemin gene is

constitutively overexpressed by the 35S cauliflower mosaic virus promoter. Prosystemin is the precursor of systemin, which initiates a signaling pathway that leads to synthesis of JA from linolenic acid (Ryan, 2000). The constitutive induction of the JA pathway in *35S::prosystemin* plants results in the systemic accumulation of high levels of PIs in these plants (McGurl et al., 1994).

As shown in Figure 4A, *Fol007* infected *def1* and *35S::prosystemin* lines both became more chlorotic than the wild-type parental cultivar Castlemart. Inoculation with the less pathogenic *FolΔAvr2* mutants did not result in obvious disease symptoms in any of the lines. Although *def1* and *35S::prosystemin* plants exhibited a slightly higher disease

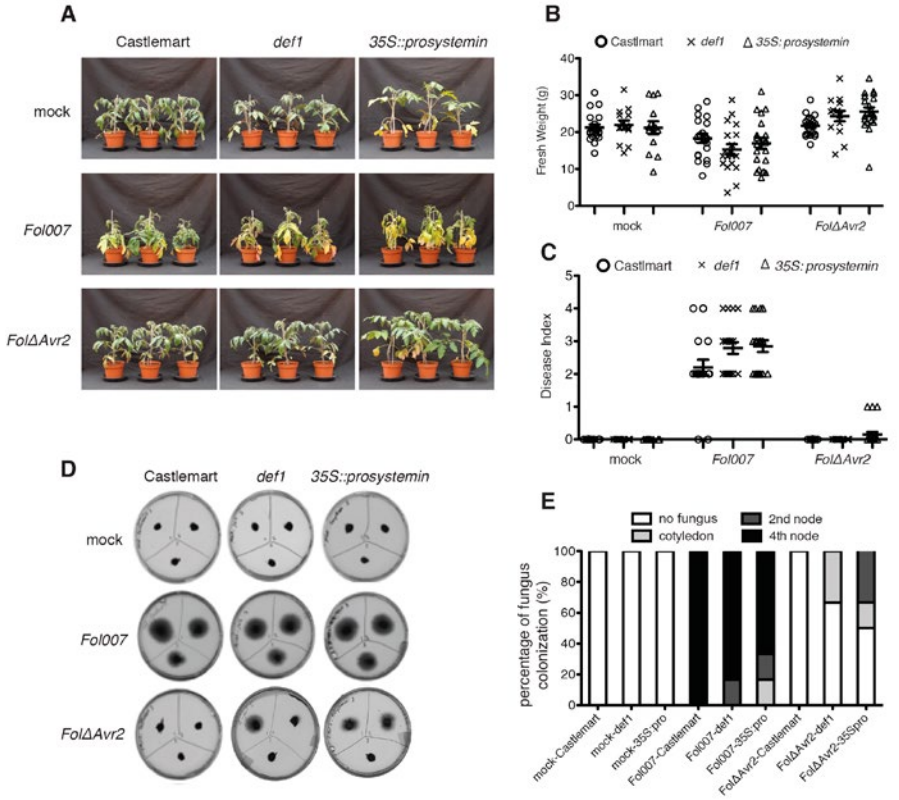

Figure 4. JA signaling is affects the ability of the fungus to colonize tomato. (A). Three-week-old wild-type Castlemart, *def1* and *35S:prosystemin* tomato plants inoculated with water (mock), *Fol007* or *FolΔAvr2* at 21 dpi. Disease symptoms were scored by measuring (B) fresh weight and (C) disease index (ranging from 0-4). Circle, × and Δ indicate Castlemart, *def1* and *35S:prosystemin* (n = 20), respectively. Plant weight and disease index were subjected to one-way ANOVA with Dunnet's *post-hoc* test (*P<0.05; ** P<0.01; ***P<0.001). The bioassay was repeated three times with similar results. (D). Stem sections from cotyledon (top-left), 2nd node (top-right) and 4th node (bottom-center) of individual plants (n=6) after five days of incubation on CDA plates. (E). Colonization is expressed as the percentage of infected slices of all stem pieces (n=6).

index and a reduction in fresh weight as compared to wild-type plants, this difference was not significant for neither *Fol007* nor *FolΔAvr2* infection (Figure 4B and C). Fungal recovery assay showed that in wild-type Castlemart *Fol007* was present in all probed stem sections, indicating that the fungus has colonized the entire stem until the forth node (Figure 4D and E). In 20-30% of the inoculated *def1* and *35S::prosystemin* plants *Fol007* did not colonize the stem until the 4th node. Interestingly, in contrast to *Fol007*, *FolΔAvr2* completely failed to colonize wild-type Castlemart plants, which is consistent with the lack of disease symptoms in these plants. However, from the symptomless *35S::prosystemin* plants the fungus could be observed to grow out from stem sections collected either at the position of the cotyledon or of the second node in 40% of the cases (Figure 4E). Also from the *def1* mutant the fungus could be cultured from the lowest node in 30% of the plants. These data suggest that altered JA homeostasis enhances the ability of the fungus to colonize the plant, but does not significantly affects disease symptom development.

SA and ET signaling pathways act synergistically in tomato susceptibility against *Fol* infection

Since low levels of SA enhance susceptibility to *Fol*, while impaired ET signaling reduces susceptibility, we wanted to test whether SA interacts with ET in the response ofv tomato to *Fol*, and to identify which of the two pathways is dominant. To this end, the expression of *PR1a* was monitored over time (0-14 dpi) in the *ACD* ethylene synthesis mutant and wild-type upon *Fol007* infection. As shown in Figure 5A, SA marker gene *PR1a* was greatly induced in wild-type plants, but much less in *ACD* lines, clearly indicating that induction of *PR1a* following *Fol* infection requires an intact ET pathway. Subsequently, the expression of *ERF* and *ETR4* were assessed in the *NahG* line and in its parental wild-type line Moneymaker following *Fol007* inoculation. As shown in Figure 5B and C, the ET signaling pathway was strongly induced in wild-type plants, but repressed in *NahG* plants inoculated with *Fol*. The above results suggest that SA and ET signaling pathways act synergistically in tomato following *Fol* infection: impairment of one pathway compromises the activation of the other.

Discussion

Here the role of SA, ET and JA in modulating susceptibility of tomato plants to *Fol* was investigated. *NahG* plants that fail to accumulate SA were hyper-susceptible to *Fol* infection and showed severe disease symptoms and extensive fungal colonization of their xylem vessels. Together with the strong induction of the SA marker gene *PR1a*, which was not induced in the *NahG* plants, these data show that SA plays a positive role in reducing disease susceptibility. This conclusion is in agreement with chemical

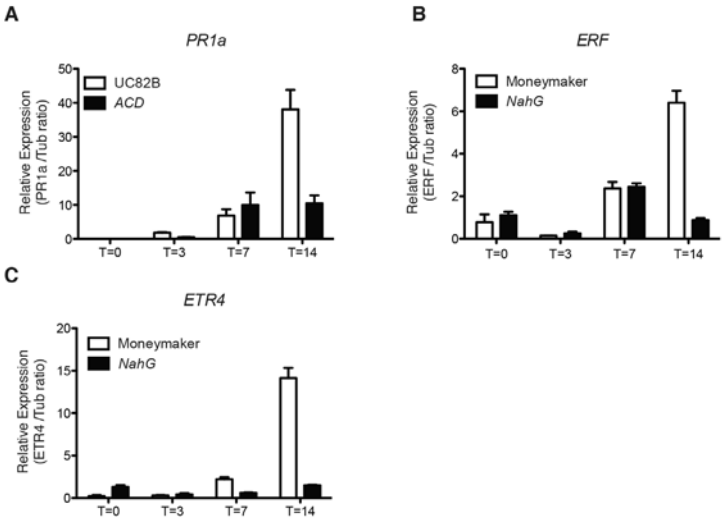

Figure 5. Time course of transcription patterns of SA and ET marker genes upon *Fol* inoculation. (A) Expression of the SA marker gene *PR1a* in wild-type UC82B and the transgenic *ACD* line at 0, 3, 7, and 14 dpi. (B-C) Transcription patterns of the ET-marker genes *ERF* and *ETR4* in *NahG* and the Moneymaker progenitor at 0, 3, 7, and 14 dpi. Gene expression levels relative to the internal control tubulin genes were quantified by qPCR.

studies, in which exogenous application of SA to tomato through root feeding or foliar sprays reduced vascular browning, leaf yellowing and wilting following *Fol* inoculation (Mandal et al., 2009). The positive role of SA in *Fo* resistance in tomato is consistent with studies with *NahG* Arabidopsis showing an increased susceptibility to *Fo* (Berrocal-Lobo and Molina, 2004, Diener and Ausubel, 2005, Thatcher et al., 2009, Trusov et al., 2009). Therefore, the role of SA in *Fo* susceptibility seems conserved in both plant species. Interestingly, in several studies, elevated SA is reported to enhance susceptibility to necrotrophic pathogens, but to promote resistance to hemibiotrophs (Bari and Jones, 2009, El Oirdi et al., 2011). Our finding in which SA reduces the susceptibility of tomato plants to *Fol* is in line with the hemibiotroph lifestyle of latter.

The *ACD* tomato line, in which ET biosynthesis is compromised, showed a reduced susceptibility to *Fol* infection. Upon *Fol* infection the transgenic line showed less disease symptoms and a reduced fungal colonization as compared to the wild-type UC82B cultivar (Figure 2). The ET marker genes *ETR4* and *ERF* were highly induced in wild-type plants, but not in the *ACD* line, indicating that ET signaling is induced in response to *Fol* infection and is important for disease development. The ET-insensitive tomato line *Nr* as well was found to be less susceptible than wild-type to infection with *Fol007* (Figure 3). These data are consistent with a study by Lund and coworkers in which a reduction of disease symptoms in the *Nr* mutant was found as well upon infection with a different *Fol* isolate (Lund et al., 1998). The fact that both ET synthesis and perception were found

to be important for disease development as well as for fungal colonization suggests that *Fol* has hijacked the host's ET signaling pathway to cause disease.

The role of ET is multifaceted in Arabidopsis (Di et al., 2016). Similar to the tomato *ethylene insensitive2* (*ein2-1*) and *etr1-1* mutants grown in soil, a reduction of disease symptoms compared to wild-type Col-0 plants is seen when inoculated with *Focn* (Trusov et al., 2009, Pantelides et al., 2013). Fo inoculation of plants carrying the *ein2-5* allele revealed a markedly enhanced susceptibility to *Fo* in plate assays (Berrocal-Lobo and Molina, 2004). These differences might be explained by the different mutations or by the different inoculation methods. The *ein2-1* mutation harbors a stop codon after the sequence encoding transmembrane domain, which might result in a partial functional EIN2, whereas the *ein2-5* mutant contains a frame-shift in the transmembrane-spanning α-helix and hence is likely a complete loss-of-function mutant (Alonso et al., 1999). As inoculation of soil-grown plants best mimics the natural infection process, which resembles that of our tomato assays, it seems that in both plant species the role of ET is conserved in that its absence reduces disease symptom development (Trusov et al., 2009, Pantelides et al., 2013).

No significant difference in disease index and fresh weight between wild-type and the JA deficient *def1* line was found after *Fol007* or *FolΔAvr2* inoculation (Figure 4). These findings contrast those of Thaler and coworkers who showed that the weight of *def1*, but not wild-type plants, was reduced upon inoculation with a race 1 *Fol* isolate (Thaler et al., 2004). One reason for the discrepancy could be the different *Fusarium* race used, or differences in assay conditions. The observation that the colonization of the vasculature of the *35S::prosystemin* plant by *Fol007* was reduced compared to wild-type plants whereas it was increased for the *FolΔAvr2* variant, indicates that Avr2 might manipulate the JA signaling pathway to promote infection. Involvement of *Fo* effectors in manipulating hormone signaling of the host has been suggested before (Di et al., 2016). Future experiments could reveal whether *Fol* effectors are indeed manipulating the JA pathway by performing bioassays on the JA mutants using different *Fol* races and/or the effector knockout strains previously described (Gawehns et al., 2015).

Upon *Fol007* infection the *35S::prosystemin* plants became slightly more chlorotic with a higher, but non-significant increase in disease index together with a minor reduction of fresh weight as compared to wild-type tomato. Fungal recovery assays revealed a more extensive fungal colonization in *35S::prosystemin* plants compared to wild-type. Thus, under our assays conditions JA appears not to play a major role in development of disease symptoms, but it facilitates fungal colonization.

In different host-*Fo* pathosystems JA can promote either resistance or susceptibility (Di

et al., 2016). A point mutation in *CORONATINE INSENSITIVE 1* (*COI1*) in Arabidopsis, an essential component for JA perception, strongly reduced susceptibility to *Fo* (Thatcher et al., 2009, Trusov et al., 2009). In contrast, *jar1* mutants that are defective in synthesis of the bioactive JA-Isoleucine conjugate showed wild-type-like symptoms or only a slight increase in susceptibility (Thatcher et al., 2009, Trusov et al., 2009). Additionally, Cole reported that infection by *Fo* f.sp. *conglutinans* and *Fo* f.sp. *matthioli*, which produce isoleucine- and leucine-conjugated jasmonate (JA-Ile/Leu), respectively is suppressed in *coi1*. In contrast, *Fo* f.sp. *raphani*, which produces no detectable JA-Ile/Leu, has no effect on infection in *coi1*. Furthermore, *Fol*, which produces no detectable jasmonates, has no effect on wilt disease in *jasmonic acid-insensitive* (*jai1*) tomato plants (Cole et al., 2014). Therefore different *formae speciales* may adopt different strategies to infect their host and to cause disease symptoms.

The SA, ET and JA signaling pathways are entangled in a complex network in which the different pathways influence each other through positive and negative regulatory interactions (Grant and Jones, 2009). We observed that compared to wild-type plants the expression of *PR1a* was reduced in the ET biosynthesis mutant *ACD* (Figure 5). Similarly, following *Fol* inoculation the induction of ET signaling, as monitored by reduced *ETR4* and *ERF* expression, was strongly decreased in *NahG* lines as compared to wild-type plants. Collectively, these results indicate that in tomato SA and ET signaling act synergistically during *Fol* infection as an intact ET pathway is required for induction of the SA reporter gene and *vice versa*. Also for *X. campestris* pv. *vesicatoria* infection of tomato accumulation of SA was found to require ET synthesis, suggesting that ET positively regulates SA-induced defenses (O'Donnell et al., 2003).

All data together allow us to propose a model for involvement of SA, ET and JA signaling in tomato toward susceptibility against *Fol* (Figure 6A). SA and ET signaling interact and have opposite roles in disease susceptibility. Infection of tomato plants by *Fol* activates both the ET and SA pathways. The ET response enhances susceptibility to *Fol* infection and disease development whereas SA responses restrict colonization. The role of JA in infection is less clear and seems mostly confined to the ability of the fungus to colonize the plants. A comparison with the reported roles of these phytohormones in Arabidopsis to *Fo* infection reveals shared and unique effects between Arabidopsis and tomato. As shown in Figure 6B, SA signaling also negatively regulates susceptibility to *Fo*, whereas ET signaling likewise positively enhances susceptibility. Notably, JA can be hijacked by the fungus to enhance pathogenicity in Arabidopsis, but only enhances the ability of the fungus to colonize tomato plants.

The role of the ET, SA and JA in susceptibility to *Fo* seems broadly conserved between *A. thaliana* and the crop tomato. This insight is relevant to allow translation of molecular

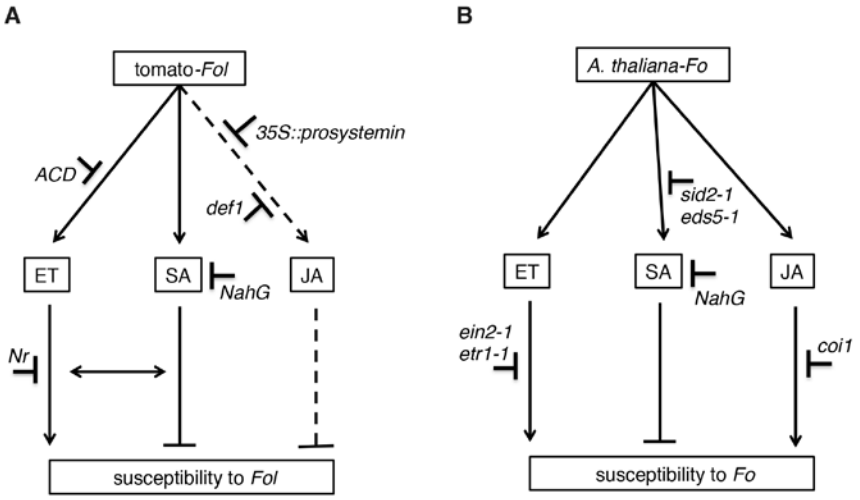

Figure 6. Proposed models for the involvement of the JA, SA, and ET signaling in tomato (A) and Arabidopsis (B) upon *Fol* infection are shown. Compromised ET biosynthesis and -perception reduce disease susceptibility, while compromised SA signaling promotes hyper-susceptibility to *Fol* infection. The SA and ET pathways act synergistically, in that induction of one pathway requires intactness of the other. JA affects the ability of the fungus to colonize tomato, but does not significantly affect disease symptom development (dashed line). By convention, the arrowhead implies positive regulation (stimulation) and the T-bar implies negative regulation.

insights obtained in this Arabidopsis model into actual crops when aiming for a reduced susceptibility to wilt disease. The molecular mechanisms underlying susceptibility, however, are currently unknown and their elucidation is a challenge for future studies.

Materials and Methods

Plant materials

Ten different tomato (*Solanum lycopersicum*) genotypes were used in these studies including the four wild-type cultivars from which these mutants are derived: Moneymaker, UC82B, Pearson and Castlemart. The transgenic *NahG* line, which is compromised in SA accumulation, is in a Moneymaker background (Brading et al., 2000). The ET-impaired mutant *ACD* (Klee et al., 1991) and the *Never ripe* mutation (Lanahan et al., 1994) are in a UC82B background and Pearson cultivar, respectively. The JA-impaired mutant *def1* (Howe et al., 1996) and prosystemin overexpressing line *35S::prosystemin* are in the Castlemart background (McGurl et al., 1994). Tomato seedlings were grown in a conditioned green house with day–night temperatures of 23–18°C and a 16h light/8h dark regime.

Fusarium inoculation assay

Wild-type *Fusarium* strain *Fol007* (race 2) and the derived *FolΔAvr2* mutant have been descripted before (Houterman et al., 2009). *Fol* strains were grown on Czapek Dox Agar (Oxoid Ltd., Basingstoke, Hampshire, UK) at 25°C for 7-10 days, Subsequently, a piece of agar carrying the fungus was transferred to 100ml minimal medium (100 mM KNO3, 3% sucrose and 0.17% Yeast Nitrogen Base without amino acids or ammonia). Conidial spores were harvested after 3-5 days of cultivation at 25 °C with shaking. After washing with sterilized water the spores were diluted to 10^6 spores/ml. For bioassay, 3-week-old tomato seedlings were uprooted from the soil. The seedlings were placed for 5 min in the Fol spore suspension (10^6 spores/ml) and subsequently potted. Disease progression was evaluated after 3 weeks. Plant weight and disease index (Gawehns et al., 2014) were scored for 20 plants/treatment.

Fungal recovery assay

Fungal colonization in tomato plants was assessed at 21 days after inoculation. Stem sections at cotyledon, 2nd node and 4th node were collected separately. The stem pieces were surface sterilized in 70% ethanol, rinsed in sterile distilled water after which the ends of the stem were removed with a sterile scalpel. Stem sections of about 5 mm thick were cut and placed on potato dextrose agar (PDA) supplemented with 200mg/l streptomycin and 100mg/l penicillin at 25°C, allowing the fungus to grow out of the stem sections. Pictures were taken after 5 of incubation at 25°C. Data were expressed as a percentage of slices showing fungal outgrowth.

Analysis of gene expression by RT-qPCR

RNA isolation and cDNA synthesis was done as described before (Gawehns et al., 2014). Briefly, total RNA from tomato stem beneath cotyledon was extracted using Trizol-Reagent (Invitrogen, Life Technologies, Grand Island, NY, U.S.A.) according to the manufacturer's instructions. The RNA was subsequently purified with RNeasy Mini kit (Qiagen, Düsseldorf, Germany) and DNA was removed by on-column treatment with RNase-free DNase(Qiagen). cDNA was synthesized using the M-MulV reverse-transcriptase RNase H minus kit (Fermentas, Thermo Scientific, Pittsburgh PA, U.S.A.). Stem tissue was collected from tomato plants 0, 3, 7, 14 days upon pathogen infection. The conditions of RT-qPCR (reverse transcription-quantitative polymerase chain reaction) experiments and the relative quantification of specific mRNA levels was performed according to Lopez-Raez et al. (2010) (Lopez-Raez et al., 2010) and using the gene-specific primers described in Table 1. PCRs were performed in an ABI 7500 Real-Time PCR system (Applied Biosystems, http://www.appliedbiosystems.com), using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). The 20ul PCR reactions contained 0.25 uM of each primer, 0.1 ul ROX reference dye and 1 ul of cDNA. The cycling program was set to 5 min at 50°C, 5 min at 95°C, 40 cycles of 15 sec at 95°C and

1 min at 60°C, followed by a melting curve analysis. The expression levels of selected genes were normalized to tomato α-Tubulin (Solyc04g077020.2) expression. Relative gene expression was calculated using the $2^{-\Delta CT}$ methods. Three biological replicates for each of the selected genes were performed.

Statistical analyses

The statistical significance of the results was determined by performing PRISM 5.0 (GraphPad, http://www.graphpad.com). The data on plant weight and disease index in SA and ET related tomato plants were subjected to a pairwise comparison with the Student's *t*-test (*P<0.05; ** P<0.01; ***P<0.001). For data on JA related tomato plants, a one-way ANOVA with Dunnet's *post-hoc* test (*P<0.05; **P<0.01; ***P<0.001) were made for each genotype. Pairwise comparisons with Student's *t*-test were also made for expression analysis for each gene in the different genotypes. All the experiments were performed twice, with similar results.

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We thank Jonathan D.G. Jones (Sainsbury Laboratory) for providing the transgenic *NahG* line. *ACD* and *Never ripe* mutants were obtained from M. Mudgett, Stanford University (provided by H. Klee, University of Florida). *def1* is obtained from M. Mudgett, Stanford University (provided by G.Howe, Michigan State University). We also thank M. Kant (University of Amsterdam) for providing *35S::prosystemin*. The authors wish to thank Ben Cornelissen for critically reading and commenting on the manuscript.

Table1. Primer sequences used in the gene expression analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
α-tubulin	TCGTGGCCACTATACCATTG	AGTGACCCAAGACCTGAACC
PR1a	TGGTGGTTCATTTCTTGCAACTAC	ATCAATCCGATCCACTTATCATTTTA
ERF	TCGTCGGGAAACGGTTCCAT	GACATCCAACTTGCATGACACTTG
ETR4	GGTAATCCCAAATCCAGAAGGTTT	CAATTGATGGCCGCAGTTG
PAL	CGTTATGCTCTCCGAACATC	GAAGTTGCCACCATGTAAGG

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Chapter 4

Uptake of the *Fusarium* effector Avr2 by tomato is not a cell autonomous event

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Abstract

Pathogens secrete effector proteins to manipulate the host for their own proliferation. Currently it is unclear whether the uptake of effector proteins from extracellular spaces is a host autonomous process. We study this process using the Avr2 effector protein from *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*). Avr2 is an important virulence factor that is secreted into the xylem sap of tomato following infection. Besides that, it is also an avirulence factor triggering immune responses in plants carrying the *I-2* resistance gene. Recognition of Avr2 by I-2 occurs inside the plant nucleus. Here we show that pathogenicity of an *Avr2* knockout *Fusarium* (*Fol*Δ*Avr2*) strain is fully complemented on transgenic tomato lines that express either a secreted (Avr2) or cytosolic Avr2 (ΔspAvr2) protein, indicating that Avr2 exerts its virulence functions inside the host cells. Furthermore, our data imply that secreted Avr2 is taken up from the extracellular spaces. Grafting studies were performed in which scions of *I-2* tomato plants were grafted onto either a *ΔspAvr2* or on an *Avr2* rootstock. Although the Avr2 protein could readily be detected in the xylem sap of the grafted plant tissues, no I-2-mediated immune responses were induced suggesting that *I-2*-expressing tomato cells cannot autonomously take up the effector protein from the xylem sap. Additionally, *ΔspAvr2* and *Avr2* plants were crossed with *I-2* plants. Whereas *ΔspAvr2/I-2* F1 plants showed a constitutive immune response, immunity was not triggered in the *Avr2/I-2* plants confirming that Avr2 is not autonomously taken up from the extracellular spaces to trigger I-2. Intriguingly, infiltration of *Agrobacterium tumefaciens* in leaves of *Avr2/I-2* plants triggered I-2 mediated cell death, which indicates that *Agrobacterium* triggers effector uptake. To test whether, besides *Fol*, effector uptake could also be induced by other fungal pathogens the *ΔspAvr2* and *Avr2* transgenic lines were inoculated with *Verticillium dahliae*. Whereas *ΔspAvr2* plants became hyper-susceptible to infection, no difference in disease development was found in the *Avr2* plants as compared to wild-type plants. These data suggest that effector uptake is not a host autonomous process and that *Fol* and *A. tumefaciens*, but not *V. dahliae*, facilitate Avr2 uptake by tomato cells from extracellular spaces.

Keywords: effector uptake, *Fusarium oxysporum*, *Verticillium dahliae*, tomato, grafting, *Agrobacterium tumefaciens*

Introduction

Microbe-secreted effector proteins enable pathogens to suppress or evade plant immunity responses, a prerequisite for successful infections. Most bacterial plant pathogens employ a type-III secretion system to directly inject type-III effector (T3E) proteins into the plant cytoplasm (Panstruga and Dodds, 2009). Fungi and oomycetes do not inject their effectors into plant cells, but secrete them into the extracellular spaces. Some fungal pathogens, such as *Cladosporium fulvum*, secrete effectors from invasive hyphae into the plant apoplast (Stergiopoulos and de Wit, 2016). Others, like *Magnaporthe grisea* and *Phytophthora infestans*, have specialized feeding structures protruding into the plant cells from which effectors are secreted (Panstruga and Dodds, 2009; Dodds and Rathjen, 2010). In either case, the effectors accumulate outside the host's plasma membrane and it is unknown how they are taken up by plant cells (Bozkurt et al., 2012; Rafiqi et al., 2012). As many effectors have been shown to act inside the host cell (Dodds et al., 2004; Armstrong et al., 2005; Catanzariti et al., 2006), there must be a mechanism by which effector proteins enter. Whether this process is a host autonomous mechanism or requires the presence of the pathogen is currently an unresolved question. To address this question its desirable to use a pathosystem in which effector secretion and action are spatially separated. In this study we focus on the fungal pathogen *Fusarium oxysporum*, which secretes its effector proteins into the xylem sap of infected plants (Houterman et al., 2007). The effectors are transported with the sap stream to exert their action in various places in the plant.

F. oxysporum is a soil-borne and highly destructive pathogen causing vascular wilt disease on a wide range of plants. The *F. oxysporum* species complex comprises different *formae speciales* (f.sp.), which collectively infect more than 100 different hosts, provoking severe losses in crops such as melon, tomato, cotton and banana, among others (Michielse and Rep, 2009). The process of infection by *F. oxysporum* can be divided into several steps: root recognition, root surface attachment and penetration, colonization of the root cortex and, in the case of wilt-inducing formae speciales, hyphal proliferation within the xylem vessels (di Pietro et al., 2003). Characteristic disease symptoms include vascular browning, leaf epinasty, stunting, progressive wilting, defoliation and eventually plant death.

In the past decades, the interaction between tomato and *F. oxysporum* f.sp. *lycopersici* (*Fol*) has evolved into an excellent model to study the molecular mechanisms underlying disease and resistance (Takken and Rep, 2010). Over 14 putative effector proteins have been isolated from the xylem sap of infected tomato plants and are called Six (for secreted in xylem) proteins (Houterman et al., 2007). For some of them, like Six1, Six3, Six5 and Six6 a virulence function has been determined, making them effectors in

sensu stricto (Rep et al., 2005; Houterman et al., 2009; Gawehns et al., 2014; Ma et al., 2015). Besides a virulence function some effectors have been found to act as avirulence determinants, triggering immune responses in resistant hosts. The relationship between Fol and tomato cultivars follows the 'gene-for-gene' hypothesis (Flor, 1971). According to this hypothesis disease resistance conferred by resistance (R) genes requires 'matching' avirulence (Avr) genes in the pathogen. Three *R* genes against *Fol* have been introgressed into cultivated tomato (*Solanum lycopersicum*): the *I* and *I-2* genes from *S. pimpinellifolium*, which confer resistance against *Fol* races 1 and 2, respectively, and the *I-3* gene from *S. pennellii*, which confers resistance to *Fol* race 3. The three *Fol* effector proteins Avr1 (Six4), Avr2 (Six3) and Avr3 (Six1), which are recognized by *I*, *I-2* and *I-3*, respectively, have all been cloned (Rep et al., 2004; Houterman et al., 2008; Houterman et al., 2009). They are secreted into the xylem sap during infection. 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 (Ma et al., 2013). Both Avr3 and Avr2 are important for pathogenicity (Rep et al., 2005; Houterman et al., 2009). Notably, Avr1 does not enhance virulence on a susceptible plant, but suppresses *I-2* and *I-3*-mediated resistance allowing the fungus to overcome the gene-for-gene resistance (Houterman et al., 2008).

Avr2 encodes a mature 15.7 kDa protein preceded by an N-terminal signal peptide. Avr2 contains two cysteine residues that might form a disulfide bond (Houterman et al., 2007). The protein appears in various positions in 2D gels of xylem sap from *Fol*-infected tomato plants, corresponding to apparent sizes from 11-14 kDa, probably as a result of proteolytic processing from the N-terminus (Houterman et al., 2007). Race 3 strains carry point mutations in *Avr2*, resulting in single amino acid changes that do not affect its virulence function but allow the protein to evade *I-2*-mediated recognition (Houterman et al., 2009). Although Avr2 is secreted into xylem sap, the Avr2 protein is recognized intracellularly in the plant nucleus by I-2 (Ma et al., 2013), implying uptake by host cells. Here we describe the generation of transgenic tomato plants expressing either full-length *Avr2* or a truncated version lacking the signal peptide encoding sequence (*ΔspAvr2*). Bioassays and grafting studies using these plants revealed that Avr2, besides it avirulence function, also exerts its virulence function inside host cells. Having an extracellular effector that is secreted in the xylem sap, but exerts its functions inside the host cell, makes this a perfect model to study effector uptake and to reveal whether uptake is a host autonomous process or requires the presence of the pathogen.

Results

Avr2 exerts its virulence function inside host cells

Avr2 was originally identified in the xylem sap of *Fol*-infected tomato plants (Houterman et al., 2007), although a nuclear localization of Avr2 is required to trigger I-2-mediated resistance (Ma et al., 2013). As yet it is unknown where in the host the protein exerts it virulence function. To identify whether Avr2 acts inside or outside host cells, transgenic tomato plants stably expressing full-length *Avr2* were generated. The expressed protein carries it's endogenous signal peptide (Figure 1A) for translocation into the endoplasmatic reticulum and subsequent secretion. Plant-produced Avr2 is, therefore expected to be secreted into the apoplastic spaces, allowing us to test whether it exerts its function extracellularly. In addition, stable transgenic plants were made expressing a truncated *Avr2* (*ΔspAvr2*, Δsp for "deletion of signal peptide") encoding the mature protein without signal peptide (Ma et al., 2013). In these plants the protein is predicted to be present exclusively in the cytosol. Expression of both full-length *Avr2* and *ΔspAvr2* was driven by the strong and constitutive CaMV 35S promoter. Both genes were fused to sequences encoding a C-terminal hemagglutinin (HA) and streptavidin-binding peptide (SBP) tag to facilitate detection of the recombinant proteins.

From kanamycin resistant T1 plants three independent transformants containing a single copy of the *Avr2* construct (*35S::Avr2-1*, *35S::Avr2-4* and *35S::Avr2-7*) and two lines containing a single copy *ΔspAvr2* lines (*35S::ΔspAvr2-3* and *35S::ΔspAvr2-30*) were identified based on their 3:1 segregation pattern. From these plants homozygous lines were produced, which were used in the subsequent assays. None of the transgenic lines exhibited morphological aberrations, or showed a phenotype distinct from non-transformed Moneymaker tomato plants when grown under standard greenhouse conditions. To determine whether the distinctively localized Avr2 effector proteins do complement the virulence defect of a *FolΔAvr2* (a *Fol Avr2* knockout; Houterman et al., 2009) strain. 10-day-old seedlings of wild-type, *ΔspAvr2* and full-length *Avr2* transgenic tomato plants were inoculated with water (mock), wild-type *Fusarium* (*Fol007*) or the *FolΔAvr2* strain. Three weeks after inoculation, mean plant weight and average disease index of 20 plants was scored. The disease index was scored on a 0-4 scale, in which 0 means that no disease symptoms developed and 4 that plants are either dead or extremely small and wilted (Gawehns et al., 2014). Moneymaker plants inoculated with *Fol007* showed severe disease symptoms such as wilting and stunting (Figure 1B shows a representative example of lines *ΔspAvr2-30* and *Avr2-4*). As observed before (Houterman et al., 2007), the *FolΔAvr2* strain is reduced in virulence as shown by the increased vigor of the plants along with higher weights and lower disease indexes as compared to *Fol007* inoculation (Figure 1C and D). Interestingly, we found that disease

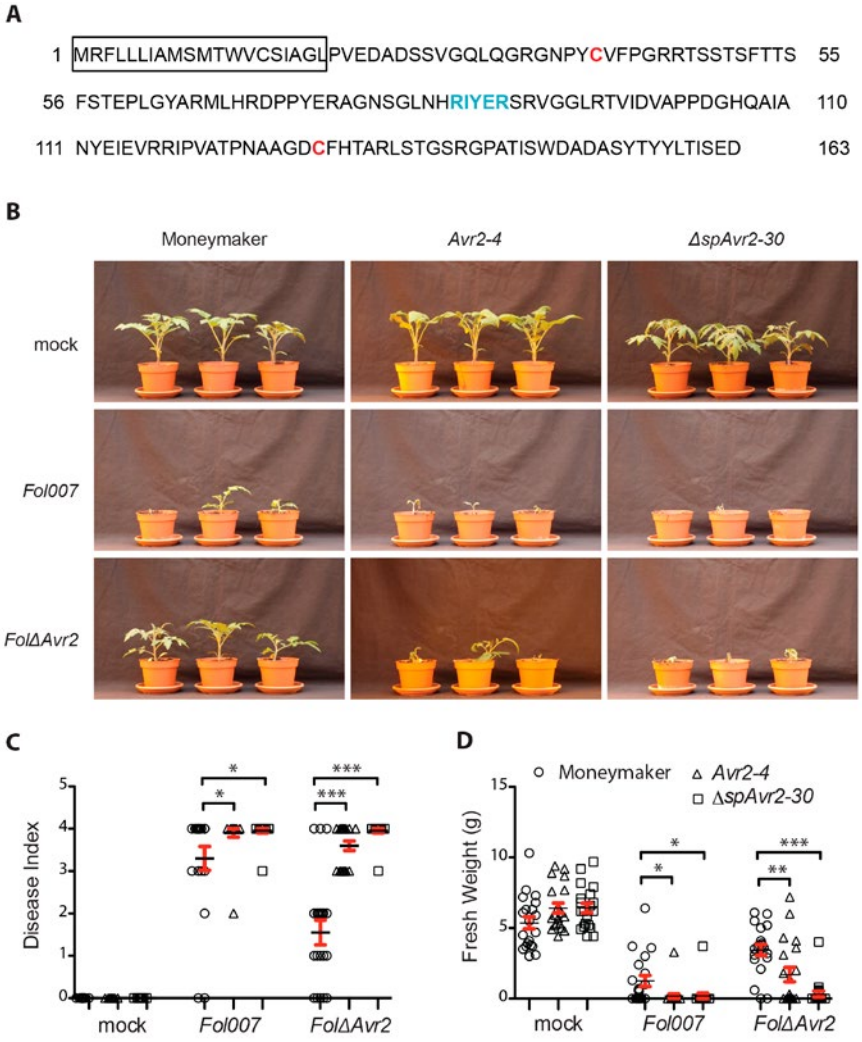

Figure 1. Avr2 exerts its virulence function inside host cells. (A) A schematic diagram of Avr2 in which the signal peptide is boxed and the two cysteine residues and the predicted RxLR (Arg-x-Leu-Arg)-like motif are marked red and blue, respectively. (B) Ten-day-old seedlings of wild type (Moneymaker), full-length *Avr2-4* and *ΔspAvr2-30* transgenic tomato plants were inoculated with water (mock), wild-type *Fusarium Fol007* or *FolΔAvr2*. Three weeks after inoculation, (C) average disease index and (D) mean plant weight of 20 plants were scored. Error bar represent means ± SE. (*P<0.05; ** P<0.01; ***P<0.001). For clarity only one representative transgenic line is shown. Data are expressed as ratio of control (vehicle condition). n>15 cells, one-way ANOVA was performed with a Bonferroni *post-test* for multiple comparison data sets, *p<0.05, **p<0.01, *** p<0.001.

symptoms of *FolΔAvr2*-infected *Avr2* plants were at least as severe as tomato plants infected with wild-type *Fol*. The regain of full pathogenicity for the *Fol Avr2* knockout strain on the Avr2 lines shows that plant-produced Avr2 effectively complements fungal virulence. Notably, also *ΔspAvr2* tomato plants infected with *FolΔAvr2* developed severe disease symptom and showed decreased plant weight and a higher disease index. Since the latter protein does not carry a signal peptide, this strongly suggests that Avr2 exerts its virulence functions inside the host cell. The experiment was performed twice using all five transgenic lines, with similar results.

In *Avr2* transgenic plants Avr2 is secreted into the xylem sap and the apoplast

To assess accumulation of Avr2 in the transgenic tomato plants, the *35S::Avr2* and *35S::ΔspAvr2* lines were subjected to immunoblot analysis using either an Avr2 specific antibody (Ma et al., 2015) or an HA antibody (Figure 2A). When probed with Avr2-specific antibody, a band with the predicted size for ΔspAvr2-HASBP (23kDa) was detected in total protein extracts from *Avr2* and *ΔspAvr2* transformants, but not in the untransformed Moneymaker control plants confirming the specificity of the antibody. In the *Avr2* transformants also one additional band of a smaller size (15kDa) was observed. The appearance of this smaller sized band suggests that Avr2 is secreted into the apoplastic spaces, after which the HA tag is cleaved by extracellular proteases (van Esse et al., 2006). The apparent weight fits the predicted size of the non-tagged Avr2 protein. When probed with HA antibody only the larger 23kDa band was detected, which indicates that the 15kDa band indeed contains the non-tagged ΔspAvr2 protein from which the tag has been removed. To determine the in planta location of the ΔspAvr2 and full-length Avr2 proteins apoplastic fluid and xylem sap were isolated from the transgenic plants. Western blot analysis of these fluids revealed that the 15kDa Avr2 protein is present in the apoplastic fluid and xylem sap of Avr2 plants (Figure 2B). Its presence in the sap shows that i) the 35S promoter is active in the mesophyll and xylem-adjacent cells and ii) the signal peptide of *Fol* is functional and iii) the protein is secreted. No Avr2 protein was detected in the extracellular fluids of *ΔspAvr2* plants, which shows that Avr2 is not secreted and hence must fulfill its virulence function inside the cell. Therefore, complementation of the compromised virulence of the *FolΔAvr2* strain in *Avr2* plants is either due to re-uptake of secreted Avr2, or due to the activity of a cytoplasmic Avr2 pool that evaded signal peptide-mediated secretion from the plant cell.

***I-2*-expressing xylem-adjacent cells do not take up Avr2 host-autonomously**

To determine whether tomato cells can take up Avr2 via a host-autonomous process, grafting studies were performed in which scions of tomato plants expressing I-2 were grafted onto wild-type Moneymaker, *ΔspAvr2* or *Avr2* rootstocks. Since Avr2 is present

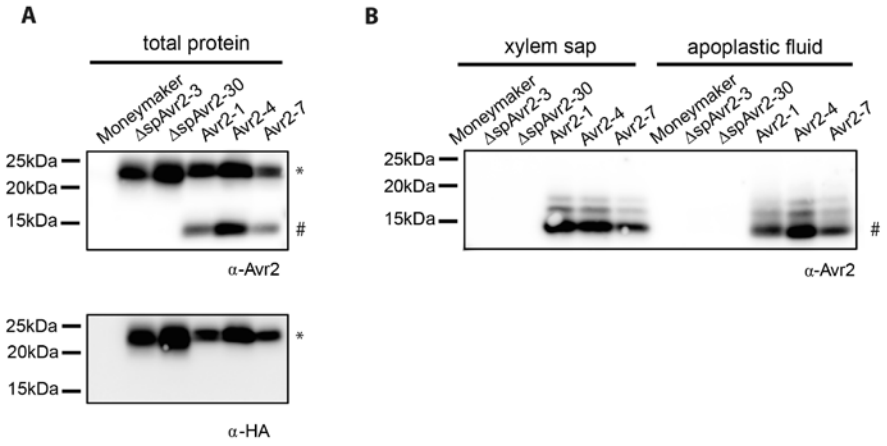

Figure 2. Avr2 accumulates in xylem sap and apoplastic fluids of *Avr2* plants. (A) Western blot showing accumulation of HASBP-tagged Avr2 in total protein extracts of transgenic tomato plants expressing either full-length *Avr2* or *ΔspAvr2*. The top blot was probed with an antibody targeted against Avr2 while the bottom blot was developed using an HA antibody. The top band (*) corresponds to the size of HASBP-tagged Avr2 whereas the lower band (#) represents the size of a non-tagged Avr2. (B) Western blot of xylem sap and apoplastic fluid isolated from the above mentioned plants probed with an Avr2 specific antibody. Avr2 accumulates in apoplastic fluid and xylem sap of transgenic tomato plants expressing full-length *Avr2*, but not in plants expressing *ΔspAvr2*. The molecular weight, as indicated by the precision plus protein standard (Bio-Rad), is shown on the left.

in the xylem sap of *Avr2* plants, through which water and nutrients are transported from roots to shoot and leaves, the effector is predicted to be transported from the *Avr2* rootstock into the *I-2* scion. If the *I-2*-expressing cells autonomously take up the effector protein from the xylem sap then I-2-mediated immune responses will be induced. As predicted, and shown in Figure 3A, no difference in growth was observed when an *I-2* scion was placed on either a wild type or a *ΔspAvr2* rootstock. The lack of growth retardation or necrosis in the chimaeras grafted on a *ΔspAvr2* rootstock is consistent with the observation (Figure 3A) that ΔspAvr2 is not secreted and hence cannot be translocated through the plant to *I-2* expressing tissues. Notably, also no I-2 immune symptoms appeared in *I-2* scions grafted on an *Avr2* rootstock (Figure 3A). Per genotype combination at least ten independent grafts were made and in none of them an autoimmune response was observed. The lack of I-2 activation suggests that either Avr2 is not transported to the upper part of the plant, or that it is not taken up from the xylem sap. To distinguish between these options western blot analysis on xylem sap was done. Xylem sap was harvested from stems cut at ±10 cm above the graft to exclude possible contamination of the sap with Avr2 leaking out of damaged *Avr2* expressing cells. As can been seen in Figure 3B the Avr2 protein could readily be detected in the

xylem sap of *I-2* scions placed on an *Avr2* rootstock, but not in xylem sap isolated from scions grafted on either wild type of an *ΔspAvr2* root stocks.

These results show that Avr2 is transported from the *Avr2* rootstock into *I-2* scions and that the absence of I-2-mediated immune elicitation is either due to inability of the plant to autonomously take up Avr2 from the xylem sap, or that the effector concentration is too low to trigger an I-2-mediated response. To examine both options non-transgenic Moneymaker and *ΔspAvr2-30* and *Avr2-7* transgenic tomato plants were crossed with *I-2* tomato plants. Combining the resistance and avirulence gene into one plant ensures a systemic presence of both proteins and a high effector abundance inside the plant. From all three crosses F1 seeds were obtained and 15 seeds per cross were analyzed for their ability to germinate. No differences in germination frequency or timing were observed. The seedlings of the different progeny were indistinguishable from each other during the first two weeks following germination. However, whereas Moneymaker/*I-2* plantlets continued to grow normally and developed into mature plants bearing fruits, the *ΔspAvr2/I-2* progeny developed a clear auto-immune phenotype; necrotic lesions emerged on the leaves and the plants showed reduced weight and stunted growth (Figure 3C). Although the *ΔspAvr2/I-2* plants continued to grow and even flowered, they never developed fruits. The autoimmune phenotype of the *ΔspAvr2/I-2* plant is consistent with the intracellular recognition of the protein by the I-2 immune receptor (Ma et al., 2013) and confirms that tomato leaf cells are capable of showing an I-2 response upon exposure to cytoplasmically localized Avr2. Therefore it was interesting to observe that no necrotic lesions developed on the *Avr2/I-2* progeny. The lack of I-2 activation in these plants implies that secreted Avr2 is not taken up from the extracellular spaces, either the xylem or apoplast, to trigger an I-2 response. The presence of the *Avr2* and *I-2* genes in *ΔspAvr2/I-2* and *Avr2/I-2* progenies was verified by PCR using *Avr2* and *I-2* specific primers on genomic DNA (Figure S1A). Additionally, western blot analysis of the leaves of parental lines and their F1 progeny (*ΔspAvr2/I-2* and *Avr2/I-2)* revealed that Avr2 is not only present in the *ΔspAvr2* and *Avr2* transgenic parental plants but also in their *ΔspAvr2/I-2* and *Avr2/I-2* progeny (Figure 3D and S1B). Taken all together, the lack of Avr2-mediated I-2 activation in *Avr2/I-2* plants suggests that *I-2*-expressing cells cannot autonomously take up the Avr2 effector protein from the extracellular spaces.

Infiltration of *Agrobacterium* in *Avr2/I-2* tomato leaves triggers HR

Previously it was reported that agro-infiltration of either an *Avr2*- or a *ΔspAvr2*-encoding construct triggers *I-2*-dependent HR in *N. benthamiana* (Houterman et al., 2009). Since the signal peptide of Avr2 is functional *in planta* (Fig 2), this finding suggests uptake of the secreted Avr2 protein by the plant cells in the presence of *A. tumefaciens*. The *Avr2/I-2* plants allow us to test this hypothesis. The expectation is that upon *A.*

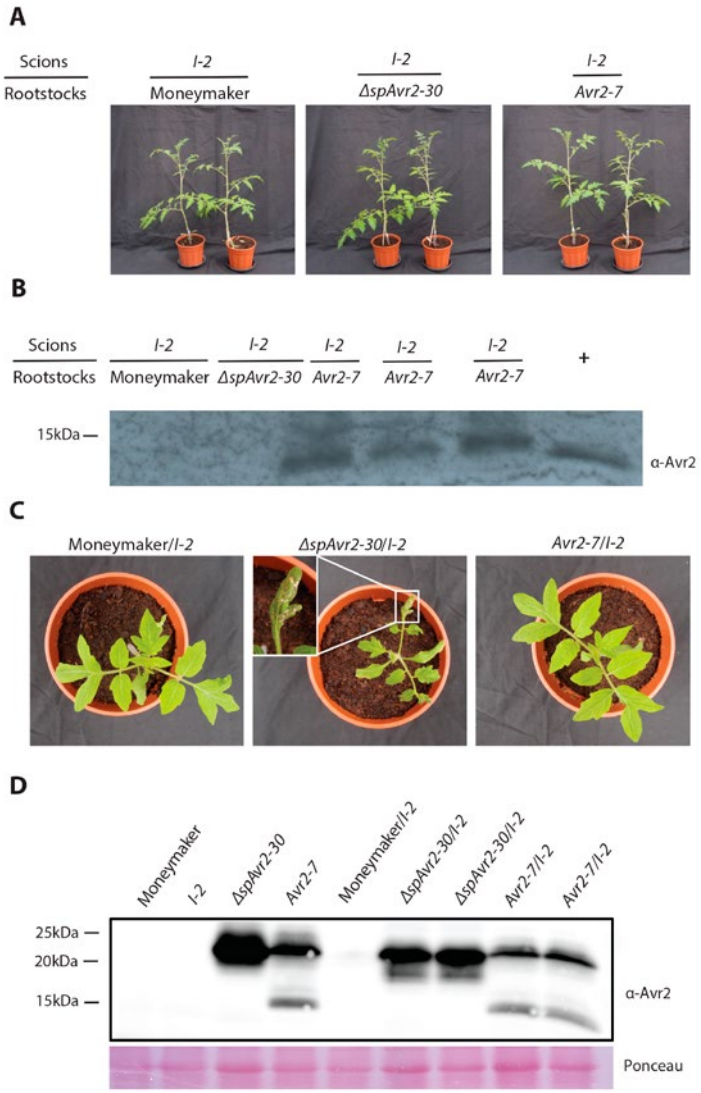

Figure 3. *I-2*-carrying tomato plants do not trigger immune signaling upon Avr2 exposure. (A) Scions of four-week-old tomato plants expressing *I-2* grafted onto a wild-type Moneymaker, a *ΔspAvr2* or an *Avr2* rootstock. Representative grafts are shown four-weeks-post grafting. Note that all grafts grew normally and did not develop autoimmune symptoms. (B) Western blot analysis of xylem sap harvested ±10 cm above the graft. The Avr2 protein could be readily detected in xylem sap of *I-2* scions placed on an *Avr2* rootstock, but not in xylem sap isolated from scions grafted on either wild-type or a *ΔspAvr2* roots stock. As a positive reference Avr2-containing xylem sap was harvested from tomato plants inoculated with *Fol007*. (C) *Avr2-7* and *ΔspAvr2-30* transformants were crossed to *I-2* tomato plants. Two weeks after germination *ΔspAvr2/I-2* plants developed clear autoimmune phenotypes; i.e. necrotic lesions, reduced plant weight and stunted growth, whereas no symptoms developed on Moneymaker/*I-2* or *Avr2/I-2* progeny. (D) Western blot analysis shows accumulation of Avr2 in Avr2 and *ΔspAvr2* transgenic tomato plants and their *ΔspAvr2/I-2* and *Avr2/I-2* progenies. The blot was probed with an antibody targeted against Avr2. Lower panel shows a Ponceau S staining that serves as loading control.

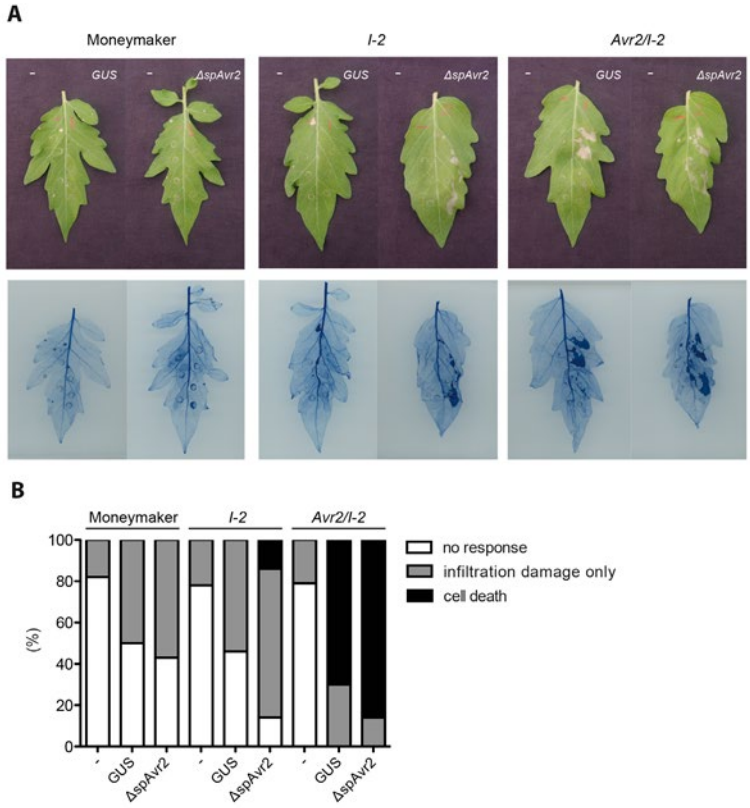

Figure 4. Infiltration of *A. tumefaciens* in *Avr2/I-2* tomato plants triggers cell death. (A) Four-week-old wild-type Moneymaker, *I-2* and *Avr2/I-2* tomato plants infiltrated with either infiltration buffer ("-") or agrobacterium expressing *GUS* or *ΔspAvr2*. The left side of each leaf is buffer infiltrated and the right site is infiltrated with agrobacterium carrying either a *GUS* or *ΔspAvr2* construct. Photographs were taken 4dpi. The bottom panel shows the same leaves stained with trypan blue to visualize cell death. (B) 20 leaves of wild-type Moneymaker, *I-2* and *Avr2/I-2* tomato plants were scored for their response following infiltration. The assay was repeated twice with similar results.

tumefaciens infiltration cell death will be triggered in the transgenic plants, but not in wild-type tomato. The *A. tumefaciens* 1D1249 strain was used to infiltrate tomato as, unlike most laboratory strains, it does not trigger necrosis in the leaf (Wroblewski et al., 2005). Four-week-old wild-type Moneymaker, *I-2* and *Avr2/I-2* tomato plants were infiltrated with *A. tumefaciens* 1D1249 delivering either *GUS*, which serves as a negative control, or *ΔspAvr2* acting as positive control for I-2 mediated cell death. To be able to distinguish specific responses from non-specific ones also a mock infiltration was done using buffer without *A. tumefaciens*. To better visualize the occurrence of cell death the leaves were stained with trypan blue. At 4 days post infiltration (dpi) the majority (80%) of mock infiltrated leaves were symptomless, although some cell death directly beneath the infiltration sites was found (Figure 4A and B). Infiltration of *Agrobacterium* delivering

either *GUS* or *ΔspAvr2* in wild-type plants also only showed cell death at the infiltration sites itself, and not in the sector around it, which can be attributed to mechanic damage. In contrast to this, agro-infiltration of *ΔspAvr2*, but not *GUS*, triggered cell death in a sector around the infiltration points in 20% of the infiltrated *I-2* tomato leaves. The induction of *I-2*-dependent cell death following transient expression of *ΔspAvr2* is consistent with the former observations in *N. benthamiana*, and shows that 1D1294 can be used for transient transformation of tomato and that tomato leaves are capable of mounting an *I-2* specific response upon Avr2 perception.

In contrast to the *I-2* plants, which only responded to an *A. tumefaciens* strain carrying *ΔspAvr2*, the majority of *Avr2/I-2* leaves exhibited a strong cell death response of the infiltrated sector following agro-infiltration of either strain. In respectively 70% and 80% of the *Avr2/I-2* leaves cell death was induced after infiltration of either *GUS* or the *ΔspAvr2* construct. The cell death is independent of the construct, but requires *A. tumefaciens* as necrosis was not induced in the mock infiltrated sectors. Together these data shows that *A. tumefaciens* infiltration triggers cell death in *Avr2/I-2* tomato plants, likely by facilitating the uptake of Avr2 by the plant cells.

Verticillium dahliae does not facilitate Avr2 uptake by tomato cells from the extracellular space to exert its intracellular virulence function

In Figure 1 it is shown that both *ΔspAvr2* and *Avr2* transgenic tomato lines fully complement the virulence defect of a *Fol ΔAvr2* knockout, implying that *Fol* facilitates Avr2 uptake from the extracellular spaces into plant cells. To test whether effector uptake is a generic phenomenon that can be induced by any vascular fungal pathogen, we tested whether *V. dahliae* can also induce effector uptake. If Avr2 targets host processes that are important for pathogenicity of *V. dahliae* then it is predicted that *ΔspAvr2* plants show hyper-susceptibility to the fungus. If so, the degree of disease susceptibility of the *Avr2* plants could be used as a proxy to monitor effector uptake.

Besides wild-type Moneymaker, two independent *ΔspAvr2*-expressing tomato lines (*ΔspAvr2-3* and *ΔspAvr2-30*) and three independent *Avr2* tomato lines (*Avr2-1*, *Avr2-4* and *Avr2-7*) were used in the *V. dahliae* bioassay. Stunting, chlorosis, necrosis and vascular browning are typical symptoms of *Verticillium* wilt disease. Hence disease symptoms following inoculation can be quantified by measuring the canopy surface and fresh weight of inoculated plants. *V. dahliae*-inoculated wild-type Moneymaker plants showed moderate stunting, as compared to mock-inoculated plants, confirming successful infection (Figure 5A). Interestingly, *V. dahliae*-inoculated *ΔspAvr2* plants were much smaller in stature and showed a significant reduction in canopy surface and fresh weight as compared to the inoculated Moneymaker plants (Figure 5B). The

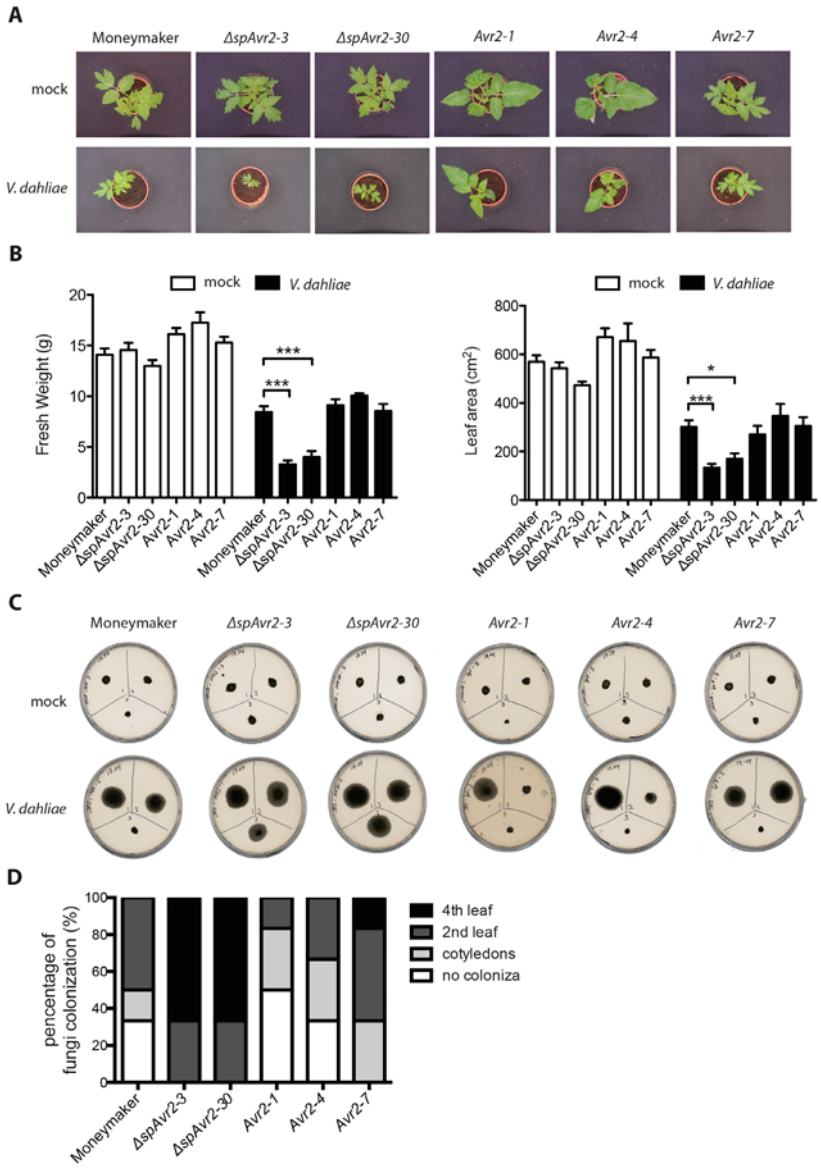

Figure 5. *V. dahliae* does not facilitate Avr2 uptake in tomato. (A) Representative pictures of mock (upper row) and *V. dahliae* race 1 JR2 (bottom row) inoculated Moneymaker, *ΔspAvr2* and *Avr2* transgenic tomato plants at 21 days post inoculation (dpi). Hyper-susceptibility was only observed in *ΔspAvr2* plants. As a measure for disease development (B) fresh weight and leaf canopy surface of inoculated plants was measured. Error bar represent means ± SE. (*P<0.05; ***P<0.001). (C) As a measure of *V. dahliae* colonization stem sections from the cotyledon (top-left), 2nd node (top-right) and 4th node (bottom-center) of each plant were collected at 21 dpi and placed on PDA plates. Pictures were taken five days post incubation on plate. (D) Fungal progression in the stem was expressed as a percentage of infected slices.

enhanced disease symptoms and hyper-susceptibility of the *ΔspAvr2* plant implies that cytoplasmatically localized Avr2 enhances the virulence of *V. dahliae* allowing us to use disease development as a proxy for effector uptake. It was, therefore, interesting to note that *V. dahliae* infected *Avr2* plants did not show hyper-susceptibility as the symptoms on these plants were indistinguishable from the non-transgenic controls. The bioassay was repeated three times with similar results (data not shown). The lack of hyper-susceptibility of *Avr2* tomato plants suggests that extracellular Avr2 is not taken up by *V. dahliae* infected plant cells in quantities sufficient to reveal its intracellular virulence-promoting activity.

To investigate in further detail whether the hyper-susceptibility in *ΔspAvr2* plants correlates with increased fungal colonization, a fungal recovery assay was performed (Fradin et al., 2009). To this end, stem sections were taken from *V. dahliae* inoculated tomato plants at positions of 1) the cotyledon, 2) the second node and 3) the fourth node, and these were placed on PDA plates and incubated for 5 days at 25°C. The fungus could only be recovered from lower stem sections of Moneymaker and *Avr2* plants whereas *Verticillium* grew out from all stem sections of *ΔspAvr2* plants (Figure 5C). *Verticillium* colonized over 60% of *ΔspAvr2* plants till the fourth node, whereas it could typically only reach the cotyledon level or second node of wild type Moneymaker and *Avr2* plants (Figure 5D). Overall, these data show that the *ΔspAvr2*, but not *Avr2* plants are hyper-susceptible towards *V. dahliae* as depicted by their enhanced fungal colonization and increased disease symptoms. These data suggest that *V. dahliae* does not facilitate Avr2 uptake by tomato cells from the extracellular spaces to exert its intracellular virulence functions.

Discussion

Here we show that expression of either full length *Avr2* or *ΔspAvr2* in tomato complements the compromised virulence of a *FolΔAvr2* strain. Hence, Avr2 not only exerts its avirulence function intracellularly (Ma et al., 2013), but also its virulence activity. How the protein exerts its virulence function is unknown, but apparently it targets a process that is also important for infection of *V. dahliae* as *ΔspAvr2* plants became hyper-susceptible to this fungus. The observation that extracellular plant-produced Avr2 fully complements the *FolΔAvr2* strain implies that the protein is either able to evade signal peptide-mediated secretion or is taken up by the plant cells. Since we detected Avr2 in xylem sap and apoplastic fluid of *Avr2* transgenic plants the signal peptide must be functional lending support to the second hypothesis. In agreement, it has been reported that Avr2 is secreted into tomato xylem vessels by *Fol* (Houterman et al., 2007), while it intracellularly activates *I-2* (Houterman et al., 2009; Ma et al., 2013) further indicating that Avr2 is taken up by plant cells in the presence of *Fol*.

How host cells take up effectors is unknown and monitoring effector movement from the pathogen to the host cell is technically challenging (Petre and Kamoun, 2014). Also our attempts to directly visualize uptake using *Fol* strains producing GFP tagged effector proteins were unsuccessful as the tags were cleaved off by extracellular proteases, like they are in this study (Fig 2A). Mostly two types of assays are used to monitor the ability of effector proteins to enter host cells; "the cell re-entry" and the "protein uptake" method (Catanzariti et al., 2006; Dou et al., 2008a; Kale and Tyler, 2011). However, both assays have their limitations (Petre and Kamoun, 2014; Lo Presti et al., 2015). The first has the drawback that it is unclear whether effectors had indeed been secreted into the apoplast prior to re-internalization, and it is therefore not possible to exclude that effectors might have escaped the secretory pathway and thus remained in the cytoplasm (Oh et al., 2009). In the second assay protein is infiltrated in leaves, or added to cell suspension in which cells are stressed or wounded, which might trigger non-specific protein internalization complicating the interpretation of the data (Yaeno et al., 2011; Wawra et al., 2013).

We have overcome the limitation of these former assays by using an unique functional assay in which effector production is spatially separated from its action, and in which no wounding is involved. Our grafting experiment shows that although Avr2 is present in the xylem sap of the *I-2* graft, and hence is translocated from the *Avr2* rootstock, it is unable to trigger *I-2*-mediated immune responses in the *I-2* scion. These data suggest that *I-2*-expressing cells do not autonomously take up the effector protein from the xylem sap in the absence of *Fol*. Crosses between either *Avr2* or *ΔspAvr2* plants with *I-2* tomato substantiate this conclusion, as an autoimmune response was only observed in the *ΔspAvr2/I-2* crosses. Furthermore, the lack of an immune response in the *Avr2/I-2* plants implies that secretion of Avr2 is a very efficient process. So although Avr2 is abundantly present in the extracellular spaces of *Avr2/I-2* progeny, the secreted Avr2 is not perceived by intracellular I-2 again implying that plant cells do not autonomously take up the Avr2 effector.

Notably, in the presence of *Fol* the secreted Avr2 protein is able to enter the host cell as it complements the virulence defect of a *FolΔAvr2* strain. This observation implies that during infection a factor is produced that is required for Avr2 uptake by the host cell. The identity of this factor is unknown, but since infiltration of *A. tumefaciens* also stimulated effector uptake, the property to generate this a factor seems to be shared by other plant pathogens. Agro-infiltration of either an *Avr2*- or a *ΔspAvr2*-encoding construct was previously shown to trigger *I-2*-dependent HR in *N. benthamiana*, suggesting uptake of secreted Avr2 in the presence of the bacterium (Houterman et al., 2009). In line with this finding, we here show that agroinfiltration of *Avr2/I-2* leaves triggers cell death irrespectively of the construct carried by the bacterium. The ID1294 strain

containing the *ΔspAvr2*-encoding construct triggers a relative weak cell death response in *I-2* tomato, which is in line with the reported low transient transformation efficiency of this strain (Wroblewski et al., 2005). In agroinfiltrated leaves of *Avr2/I-2* plants slightly more cell death was induced by the *ΔspAvr2* carrying *A. tumefaciens* strain than by the *GUS* control strain. This difference is likely attributable to a higher cytosolic Avr2 concentration due simultaneous uptake and production of Avr2 in the *Avr2/I-2* cells. Nevertheless, it is clear that the mere presence of the bacterium in *Avr2/I-2* plants is sufficient to trigger cell death. These findings are especially relevant for studies in which pathogen-independent uptake was suggested based on assays in which the effectors were expressed using *A. tumefaciens* (Rafiqi et al., 2010; Kloppholz et al., 2011).

Given that *A. tumefaciens* has the property to trigger effector uptake, it was surprising to find that *Avr2*-expressing plants did not become hyper-susceptible to *V. dahliae*, as it suggests that this pathogen does not induce effector-uptake by host cells. That Avr2 can confer hyper-susceptibility was shown by the *ΔspAvr2* plants; compared to control plants the *ΔspAvr2* plants show a significant reduction in canopy surface and fresh weight following *V. dahliae* infection. Together these results imply that *V. dahliae* either does not produce the factor that is required for effector uptake, or that the fungus does not produce it in sufficient amounts to exert a measurable effect in the bioassay. The latter would be in line with the very low amount of fungal biomass typically observed in *V. dahliae* infected plants (Faino et al., 2012). Future experiments, using other plant pathogenic fungi and bacteria, can reveal whether the ability of plant pathogens to trigger effector uptake depends on the type of pathogen, or on the amount of microbial biomass, or both. The materials described in this paper are perfectly suited to address this question.

Uptake of effectors by plants has been described before (Dou et al., 2008b; Kale et al., 2010), but it is currently unclear which properties determine whether an effector can be taken up by the host. For the AvrM and AvrL567 effectors from the flax rust fungus *Melampsora lini* it was shown that their N-termini are required for translocation into host cells when transiently expressed using *A. tumefaciens* transformation (Ve et al., 2013). For ToxA from *Pyrenophora tritici-repentis* the C-terminal RGD motif is involved in internalization (Manning et al., 2008). The Avr2 protein does not show sequence homology with the flax rust effector proteins, nor does it contain a clearly distinguishable RGD motif. However, it has been proposed that Avr2 contains an RxLR (Arg-x-Leu-Arg)-like motif (Figure 1A) that might be involved in its uptake (Kale et al., 2010). The RxLR and DEER motifs (Asp-Glu-Glu-Arg) are frequently found in oomycete effectors (Bhattacharjee et al., 2006; Tyler et al., 2006; Jiang et al., 2008) and have been shown to function as a host-targeting signal allowing the protein to be translocated into host cells (Whisson et al., 2007; Dou et al., 2008a). Kale et al. (2010) reported

that the RxLR motif allows effectors to bind phosphatidylinositol-3-phosphate (PI3P) present on the outer surface of the plant plasma membrane enabling vesicle-mediated endocytosis. However, whether the RxLR-like motif in Avr2 is functional and required for uptake remains unclear as mutations in the motif rendered the protein unstable when transiently expressed in the plant prohibiting functional analysis (Ma, 2012).

Samuel and co-workers (2015) proposed that effectors could be transferred by extracellular vesicles (EVs). Proteins lacking secretion signals could be packaged into EVs for passage through the plasma membrane whilst proteins containing a secretion signal could be secreted into the matrix of the cell wall and then bind to EVs via a lipid binding motif. The protein then transits the cell wall as a passenger on the outer leaflet of the vesicle (Samuel et al., 2015). Whether such a mechanism applies to Avr2 is unclear. An indication that the effector might associate with vesicles is the observation that RFP-tagged Avr2 expressed from *Fol* during colonization of the xylem vessel forms red-fluorescent punctate spots alongside the mycelium where it touches the plant cells (Ma, 2012). A local high concentration of the RFP-labeled effector is consistent with the protein being sequestered on a specific location from where it could be internalized into vesicles. How an effector protein subsequently dissociates from a vesicle and is released in the host cytoplasm remains unclear; possibly the unknown factor produced during infection plays a determining role in this process. The nature of the factor triggering effector uptake is unknown. It might be another secreted effector protein from the pathogen, a microbial metabolite or a plant signal generated upon pathogen-inflicted damage. Since both *A. tumefaciens* and *Fol* trigger effector uptake we favor the latter option, also because the "protein uptake" method shows effector uptake by *in vitro* grown cultured plant cells in the absence of a microbe (Dou et al., 2008a; Kale and Tyler, 2011). Future studies are required to reveal the identity of this elusive factor.

Conclusion

we here describe a series of functional assays demonstrating that tomato cells do not take up the *Fol* Avr2 effector protein in the absence of a plant pathogen. Effector uptake was shown in the presence of both *Fol* and *A. tumefaciens.* The *Avr2/I-2* tomato plants generated in this study provide an excellent starting point to investigate whether other plant pathogens also have the ability to trigger effector uptake, and to identify the factor responsible for this process.

Materials and Methods

Plant material and fungal and bacterial strains
Tomato (*Solanum lycopersicum*) cultivar Moneymaker, which is susceptible to *Fol* race

2 *Fol007*, and a resistant cultivar, 90E341F, which contains the *I-2* resistance locus were used (Stall and Walter, 1965; Kroon and Elgersma, 1993). Tomato plants were germinated and grown on soil with 16/8 h light/dark cycles, at 22/16°C day/night and 70% relative humidity in a temperature-controlled green house. *FolΔAvr2* carrying a deletion of the *Avr2* gene in the *Fol007* background was described previously (Houterman et al., 2009).

Construction of binary vectors

Full length *Avr2* was PCR-amplified with primers FP2524 (5′-CGCTCTAGAATGCGTTTCCTTCTGCTTAT-3′) and FP2274 (5′-GCGGGATCCTCCATCCTCTGAGATAGTAAG-3′) using CTAPi::*Avr2* as template (Houterman et al., 2009). The obtained products were cloned into the vector pSLDB3104 (Tameling et al., 2010) between the *Xba*I and *Bam*HI restriction sites to generate SLDB3104::*Avr2*. SLDB3104::*ΔspAvr2* has been described before (Ma et al., 2013). All PCR primers were purchased from MWG (http://www.mwg-biotech.com) and sequences of all plasmids were confirmed by sequence analysis. *Avr2* and *ΔspAvr2* were cloned behind the cauliflower mosaic virus 35S promoter for constitutive expression and fused to a C-terminal hemagglutinin (HA) and streptavidin-binding peptide (SBP) tag. The resulting vector was introduced by electroporation into LBA4404 (Hoekema et al., 1983) for tomato transformation.

Plant transformation

Moneymaker was transformed with the construct described above using *Agrobacterium*-mediated transformation as described before (Cortina and Culianez-Macia, 2004). Briefly, surface-sterilized seeds were sown on Murashige and Skoog (MS) agar supplemented with sucrose (15g/l). The seeds were incubated in the dark in a growth chamber at 25°C for 2 days, and subsequently exposed to light. After 10 days, the base and the tip of the cotyledons was removed and the cotyledons were placed upside up in Petri dishes containing co-cultivation medium (MS agar supplemented with 30g/l sucrose, 0.5g/l 2-(N-orpholino) ethanesulfonic acid (MES) [Duchefa] and 0.2mM Acetosyringone, pH 5.75). The plates were incubated for 24 hours at 25°C in dark. Transgenic A. tumefaciens carrying the construct of interest was grown in 30ml LBman at 28°C overnight (max 16-18 hours). After harvesting, the bacteria were resuspended in 30ml LM2 medium (4.4g/l MS, 30g/l sucrose, 0.5g/l MES [Duchefa] and 0.2mM Acetosyringone, pH 5.75). Subsequently, the explants were incubated in the bacterial suspension for maximal 1 minute, briefly dried on sterile filter paper and placed on co-cultivation plates. The plates were incubated in the dark for 48 hours at 25°C after which the explants were transferred to selection plates (MS agar supplemented with 30g/l sucrose, 0.5g/l MES, 0.5mg/l zeatin riboside, 0.5mg/l indole-3-acetic acid (IAA), 250mg/l carbenicilline, 100mg/l vancomycin, and 40mg/l kanamycin, pH 5.75). Explants were transferred to fresh selection plate every two weeks. When callus appeared, it was transferred to

new selection plates until shoots appeared. Upon shoot development, the shoots were harvested and transferred to root-inducing medium (MS agar supplemented with 15g/l sucrose, 0.5g/l MES, 4g/l gelrite, 50mg/l kanamycin, pH 5.75). Once roots developed, the plantlets were potted in soil and transferred to the greenhouse where they were grown under standard greenhouse with conditions of a 16h photoperiod and 70% relative humidity at 25°C.

First-generation transformants of *ΔspAvr2* and *Avr2* were selected on 1/2 MS medium containing kanamycin (40mg/l). For the *ΔspAvr2* transgenic line, 25 seeds of nine T1 progeny were analyzed for segregation by scoring the ratio of kanamycin-resistant to kanamycin-sensitive seedlings. Six lines segregated roughly 3:1 for green versus yellowing seedlings. Subsequently the kanamycin-resistant plants were transferred to soil and self-fertilized. Homozygous single insertion lines were selected from the kanamycin resistant T2 plants according to their segregation pattern. Of each independent T2 line 25 plants were checked by PCR with primer pairs FP962 (5′-TGAGCGGGCTGGCAATTC-3′) and FP963 (5′-CAATCCTCTGAGATAGTAAG-3′) detecting a 273-bp fragment of the *Avr2* gene. Two lines were homozygous for the *Avr2* transgene (*ΔspAvr2-3* and *ΔspAvr-30*). Homozygous *Avr2* transgenic lines were screened using the same procedure. Eventually three of 23 *Avr2* plants (*Avr2-1*, *Avr2-4* and *Avr2-7*) were kept for further study. Primer pairs FP962 and FP963, and FP484 (AAAGCGTGGTATTGCGTTTC) and FP165 (TTCCGGATGTCCCATAGGATCC) were used to amplify *Avr2* and *I-2* from genomic DNA of *Avr2/I-2* and *ΔspAvr2/I-2* plants, respectively.

Protein extraction and western blotting

Protein extraction was done as described previously (Ma et al., 2015). To verify presence of Avr2 in transgenic tomato plants, leaves were harvested and snap-frozen in liquid nitrogen. After grinding the tissue with a mortar and a pestle, the powder was allowed to thaw in 2 ml protein extraction buffer per gram of tissue (25mM Tris pH 8, 1mM EDTA, 150mM NaCl, 5mM DTT, 0.1% NP-40, 1 Roche complete protease inhibitor cocktail (http://www.roche.com) and 2% PVPP). Extracts were centrifuged at 12,000 g, 4°C for 10min, and the supernatant was passed over four layers of Miracloth (http://www.merckmillipore.com/NL/en/product/Miracloth,EMD_BIO-475855) to obtain a "total" protein lysate. 20ul samples were mixed with Laemmli sample buffer and were run on 13% SDS–PAGE gels and blotted on PVDF membranes using semi-dry blotting. Skimmed milk powder (5%) was used as a blocking agent. The membranes were subjected to immunoblotting using anti-Avr2 antibody (1:10,000 diluted) (Ma et al., 2015). The secondary antibody goat-anti-rabbit (P31470, Pierce) was used at a 1:5000 dilution. The luminescent signal was visualized by ECL using BioMax MR film (http://www.sigmaaldrich.com/catalog/substance/carestreamkodakbiomaxmrfilm123459876

5?lang=en&region=NL).

Isolation of apoplastic fluid from tomato leaf tissue

Apoplastic fluid of tomato plants was isolated as described (Joosten, 2012). Four-week-old fully stretched tomato leaves or leaflets were harvested and placed in a beaker with sterile water. The beaker was placed in a vacuum desiccator and a mild vacuum was employed using a vacuum pump. While slowly releasing the vacuum by opening the vent on the desiccator jar, the leaf tissue became water-soaked and dark in color. The infiltrated leaves were gently dried using tissue papers and then rolled up and placed in a 20ml syringe hanging in a 50ml tube. Apoplastic fluid was isolated by centrifuging at 1,000g for 10 min at 4°C. For electrophoresis 20ul of collected apoplastic fluid was mixed with Laemmli sample buffer and separated on a 13% sodium dodecyl sulfate (SDS) polyacrylamide gel.

Xylem sap collection from tomato

Xylem sap was collected as described (Rep et al., 2002; Krasikov et al., 2011). Briefly, stems of six-week-old tomato plants were cut below the second true leaf and the plant was placed in a horizontal position. Then, for minimal 6h sap bleeding from the cut surface was collected in tubes placed on ice. For electrophoresis 20ul of collected xylem sap was mixed with Laemmli sample buffer and after heating separated on a 13% sodium dodecyl sulfate (SDS) polyacrylamide gel.

Fusarium inoculation assay

Fol was grown in minimal medium (100mM KNO3, 3% sucrose and 0.17% Yeast Nitrogen Base without amino acids or ammonia) and spores were harvested after 3-5 days of cultivation at 25°C with shaking. After washing with sterilized water the spores were diluted to 10^7 spores/ml. For bioassay, ten-day-old tomato seedlings were uprooted from the soil. The seedlings were placed for 5 min in the *Fol* spore suspension (10^7spores/ml) and potted. Disease progression was evaluated after three weeks. Plant weight and disease index (Gawehns et al., 2014) were scored for 20 plants/treatment. Using PRISM 5.0 (GraphPad, http://www.graphpad.com) a pairwise comparison for plant weight was done using the Student's *t*-test and disease index data was analyzed using a nonparametrical Mann-Whitney *U*-test.

Agrobacterium-mediated transient transformation in tomato leaves

The binary ctapi::*GUS* and ctapi::*ΔspAvr2* constructs (Houterman et al., 2009) were transformed into *A. tumefaciens* 1D1249 (Wroblewski et al., 2005). *Agrobacterium*-mediated transient transformation was performed as described (Ma et al., 2012). Briefly, the agrobacteria were grown to an absorbance of 0.8 at 600nm in LB-mannitol medium (10g/l tryptone, 5g/l yeast extract, 2.5g/l NaCl, 10g/l mannitol) supplemented with

20uM acetosyringone and 10mM MES pH 5.6. Cells were pelleted by centrifugation at 4000g at 20°C for 10 min and then suspended in infiltration medium at an absorbance of 0.5. (1×MS salts, 10mM MES pH 5.6, 2% sucrose, 200uM acetosyringone). Infiltration was done in four-week-old tomato leaves.

Trypan blue staining

Leaves were boiled for 5 min in a 1:1 mixture of 96% ethanol and staining solution (100ml lactic acid, 100ml phenol, 100ml glycerol, 100ml H2O and 100mg Trypan bule). The leaves were destained in 2.5g/ml chloral hydrate in water (Ma et al., 2012).

V. dahliae inoculation assay

Ten-day-old tomato plants were carefully uprooted from the soil and the roots were placed in a race 1 *V. dahliae* JR2 inoculum (1×106 conidia/ml) for 5 min (Fradin et al., 2009). Thereafter, the plants were transferred to fresh soil. After two weeks disease symptom were scored by measuring the canopy surface and fresh weight of the plants (Fradin et al., 2009). A one-way ANOVA with Dunnet's *post-hoc* test for weight and leaf area was performed using PRISM 5.0. Fungal colonization in tomato plants was assessed at 21 dpi. Stem sections at the position of the cotyledon, 2nd node and 4th node were collected separately. The stem pieces were surface-sterilized in 70% ethanol, rinsed in sterile distilled water, and the ends of the stem that had been exposed to the water were removed with a sterile scalpel. Stem sections of about 5 mm thick were cut and placed on potato dextrose agar (PDA) plates supplemented with 200mg/l streptomycine and 100mg/l penicillin at 25°C, allowing the fungus to grow out of the stem sections. Pictures were taken after five days of incubation at 25°C. Data are expressed as a percentage of infected slices.

Grafting

Four-week-old rootstocks and scions represent the best stage for grafting (http://horticulture.ucdavis.edu/main/Deliverables/Kleinhenz/tomato_grafting_guide.pdf). A similar diameter of the stem of the rootstock and scion increases the likelihood that their vasculatures align after grafting. The rootstock plant was cut between the cotyledons and first true leaf. The scion plant was cut at the same position at the main stem. Leaves from the scion were trimmed to reduce water loss. The stump of the scion seedling was cut to fit the shape of a two-sided wedge. Approximately one-third of each side was removed at a roughly 45° angle. The stump of the scion seedling was trimmed on both sides, creating a wedge with angled sides of approximately 45°. The wedge-shaped scion stump was inserted into the cut of the bisected rootstock stump. Parafilm was used to fix the rootstock and scions and to secure the graft. Grafted plants were placed for 5 days in a growth chamber with high humidity to reduce dehydration stress and increase the survival rate.

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Supplementary data

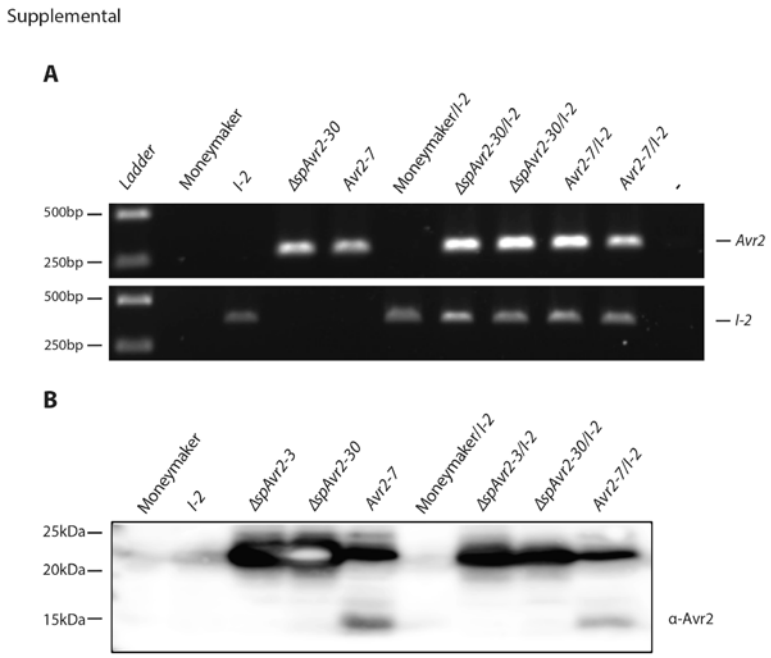

Figure 1. Presence of *Avr2* and *I-2* gene in *ΔspAvr2/I-2* and *Avr2/I-2* tomato plants. (A) Ethidium bromide stained agarose gel showing the PCR products obtained with either *Avr2* or *I-2* specific primers using DNA extracted from the indicated plants. The GeneRuler 1kb DNA Ladder (Fermentas) is shown on the left. (B) Western blot analysis shows accumulation of Avr2 in the parental *Avr2* and two independent *ΔspAvr2* transgenic tomato plants, and in two independent *ΔspAvr2/I-2* and *Avr2/I-2* progenies. The blot was probed with an antibody targeted against Avr2. The precision plus protein standard (Bio-Rad) is shown on the left.

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Chapter 5

The *Fusarium oxysporum* Avr2 effector compromises the PAMP-triggered immune response in tomato

All data in this chapter have been submitted as:

Di, X., Cao, L., Tintor, N., Hughes, R. K., Banfield*, M. J., Takken, F. L. W.* (2017) Structure-function analysis of the *Fusarium oxysporum* Avr2 effector allows uncoupling of its immune-suppressing activity from recognition. * Equal contribution

Abstract

Fusarium oxysporum f.sp. *lycopersici* (*Fol*) causes tomato wilt disease. Upon colonization the fungus secretes the Avr2 effector protein into the xylem sap. Besides being a virulence factor, Avr2 triggers immune responses in plants carrying the *I-2* resistance gene. Strains that evade *I-2* recognition carry specific point mutations in *Avr2* (e.g. the *Avr2*^{R45H} variant) that compromise avirulence, but leave virulence unaltered. To obtain a better insight in these distinct activities at the molecular level, several studies were undertaken. Transgenic tomato plants expressing either wild-type *ΔspAvr2* (lacking the signal peptide encoding sequence) or the *ΔspAvr2*^{R45H} variant both fully complemented the virulence defect of *FolΔAvr2*, showing the functionality of the plant-produced effector. Notably, *ΔspAvr2* plants became hyper-susceptible to *Verticillium dahliae*, *Pseudomonas syringae* and *Botrytis cinerea*. Consistent with the idea that Avr2 targets a conserved defense mechanism, we found that *ΔspAvr2* transgenic tomato plants were attenuated in pathogen-associated molecular pattern-triggered immunity (PTI): flg22-induced growth inhibition, reactive oxygen species (ROS) production and callose deposition were all greatly alleviated in *ΔspAvr2* plants. Structure-guided mutagenesis allowed uncoupling the virulence from the avirulence function: mutants Avr2$^{T145E/K}$ and Avr2^{T53R} were found to be impaired in PTI suppression, but retained their avirulence function. As distinct regions are involved, this implies that I-2-mediated recognition is not based on monitoring Avr2 virulence activity.

Introduction

Plants have evolved a two-layered immune system to halt pathogens (Chisholm et al., 2006; Jones and Dangl, 2006). First, the recognition of specific conserved pathogen-associated molecular patterns (PAMPs) by plant pattern recognition receptors (PRRs) at the surface of the plant cell initiates the PAMP-triggered immune (PTI) response (Jones and Dangl, 2006; Bent and Mackey, 2007; Boller and Felix, 2009; Dodds and Rathjen, 2010; Schwessinger and Ronald, 2012). One of the best-characterized PAMP/PRR pairs is the FLAGELLIN SENSING 2 (FLS2) receptor mediating recognition of bacterial flagellin (or the elicitor-active peptide flg22 derived thereof). In the presence of the ligand FLS2, a leucine-rich repeat receptor-like kinase (LRR-RLK), forms a complex with the BRI1-Associated Receptor Kinase 1 (BAK1) (Boller and Felix, 2009). Subsequently, the BAK1-FLS2 complex constitutively associates with BOTRYTIS-INDUCED KINASE 1 (BIK1), which becomes rapidly phosphorylated and released from the complex to activate downstream immune responses (Lu et al., 2010; Zhang et al., 2010). PTI comprises different cellular responses that fall into two categories, notably early and late (Boller and Felix, 2009). Early PTI responses include the rapid and transient production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases, transcriptional reprogramming and stomatal closure (Melotto et al., 2006). Late PTI responses such as callose deposition or seedling growth inhibition develop over longer time periods ranging from hours to days (Nicaise et al., 2009). Successful pathogens are able to overcome PTI by the production of virulence factors - called effectors - that can inhibit PRR complexes or their downstream signaling events. To halt such pathogens plants evolved a second class of receptors that recognizes effectors or their action, and subsequently induces effector-triggered immunity (ETI). For example, the type III secreted effector (T3SE) HopZ1a is an acetyltransferase of the pathogen *Pseudomonas syringae* that elicits effector-triggered immunity (ETI) when recognized in *Arabidopsis thaliana* by the nucleotide-binding leucine-rich repeat (NB-LRR) protein ZAR1 (Lewis et al., 2013).

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 host species, each forma specialis (f.sp.) of *Fo* is specific to a unique host (Michielse and Rep, 2009). Of all *Fo* pathosystems the interaction between *Fo* f.sp. *lycopersici* (*Fol*) and tomato is among the best studied, and over last decades it evolved into an excellent model to study the molecular mechanisms underlying disease and resistance (Takken and Rep, 2010). *Fol* attaches to the root surfaces of its host, penetrates and subsequently colonizes the plant xylem vessels (di Pietro et al., 2003). Subsequent blockage of the vasculature prevents transport of water and nutrients causing the typical wilt symptoms after which

this disease is named.

To date, three resistance (R) genes, notably *I*, *I-2* and *I-3* have been introgressed from wild tomato species into cultivated tomato (*Solanum lycopersicum*) to confer resistance against *Fol* races 1, 2 and 3, respectively. *Fol* effector proteins occurring in the xylem sap of infected tomato plants are called Six (Secreted in xylem) proteins (Rep et al., 2004; Houterman et al., 2008). Some of the Six proteins have been designated Avirulence (Avr) proteins as well, since they are recognized by matching R proteins of the host. Six1 (Avr3) is a virulence factor that triggers *I-3*-mediated resistance. Six4 (Avr1) induces *I*-mediated resistance and suppresses I-2- and I-3-mediated disease resistance (Rep et al., 2005; Houterman et al., 2008). Six3 (Avr2) is required for virulence in susceptible tomato, but triggers resistance in plants carrying the resistance gene *I-2* (Houterman et al., 2009). Race 3 strains carry point mutations in *Avr2* that do not affect its virulence function but allow these strains to evade *I-2* mediated recognition (Houterman et al., 2009; Chellappan et al., 2016). *Avr2* encodes a protein with a signal peptide at its N terminus and the 15.7 kDa mature protein carries two cysteines (Houterman et al., 2007). *I-2* encodes a classical nucleotide binding-leucine rich repeat (NB-LRR) type resistance protein, carrying an N-terminal coiled-coil (CC) domain (Simons et al., 1998; van Ooijen et al., 2007). In contrast to the conserved structural similarity of plant R proteins, Avr2 does not share sequence homology to known proteins (Houterman et al., 2009), and hence it is difficult to predict its possible biochemical functions based on the primary protein sequence.

To gain more insight in the virulence activity of Avr2 *in planta*, we constitutively expressed a cytosolic version of the wild-type *Avr2* and the *Avr2*^{R45H} variant in tomato. Avr2^{R45H} is a *Fol* race 3 variant of wild-type Avr2 that losts its avirulence function, allowing race 3 isolates to infect resistance gene *I-2* containing tomato (Houterman et al., 2009). Here, we report that heterologous expression of *ΔspAvr2* and *ΔspAvr2*^{R45H} promotes susceptibility towards various plant pathogens. In line with this observation we demonstrate that Avr2 suppresses PTI responses; flg22-induced growth inhibition, ROS production and callose deposition are all greatly alleviated in *ΔspAvr2* plants. Structure-guided site directed mutagenesis of Avr2 combined with functional analysis of the mutants, allowed us to identify residues that are critical for the effector's virulence function and to uncouple it from its avirulence activity.

Results

Expression of *Avr2* in tomato confers hyper-susceptibility to *Verticillium dahliae*

Previously it has been shown that plant-produced Avr2 protein translated from *Avr2*

lacking the signal peptide-coding sequence (*ΔspAvr2*), effectively complements fungal virulence of a *FolΔAvr2* knockout strain showing that the protein acts inside the cell (Di et al., 2016). To test whether the virulence promoting activity of Avr2 extends to other xylem-infecting fungi, susceptibility of *Avr2*-expressing tomato plants towards *Verticillium dahliae* was assessed. Thereto *ΔspAvr2* and *ΔspAvr2^{R45H}* transgenic tomato plants were inoculated with spores of a race isolate 1 of *V. dahliae* (strain JR2). Besides wild-type Moneymaker, two independent *ΔspAvr2*-expressing tomato lines (*ΔspAvr2-3* and *ΔspAvr2-30*) and two *ΔspAvr2^{R45H}* expressing tomato lines (*ΔspAvr2^{R45H}-1* and *ΔspAvr2^{R45H}-11*) were tested. Stunting, chlorosis, necrosis and vascular browning are typical symptoms of *Verticillium* wilt disease. Hence to quantify disease symptoms the canopy surface of inoculated plants was measured. *V. dahliae*-inoculated Moneymaker plants showed moderate stunting when compared with mock-inoculated plants (Figure 1A). *V. dahliae* inoculated *ΔspAvr2* and *ΔspAvr2^{R45H}* plants, however, became hyper-susceptible: they showed severely stunted growth and a significant reduction in canopy surface when compared to the inoculated Moneymaker plants (Figure 1A and 1B).

To investigate whether the hyper-susceptibility correlates with increased fungal colonization, a fungal recovery assay was performed (Fradin et al., 2009). Stem sections

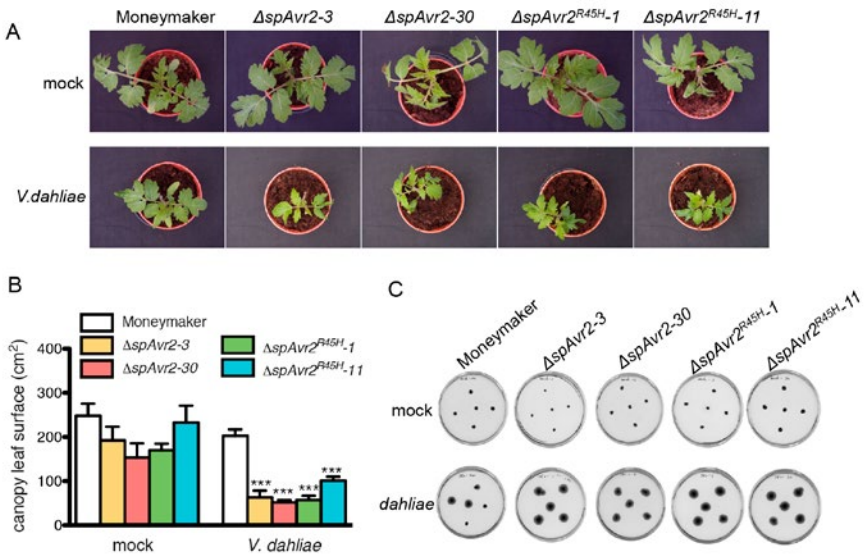

Figure 1. *ΔspAvr2* and *ΔspAvr2^{R45H}* transgenic tomato plants show enhanced susceptibility to *V. dahliae*. (A) Representative pictures of mock (upper row) and race 1 JR2 (bottom row) inoculated Moneymaker and *ΔspAvr2* and *ΔspAvr2^{R45H}* transgenic tomato plants at 21 days post inoculation (dpi). (B) As a measure for disease severity, leaf canopy surface of inoculated plants was measured. Error bar represents means standard deviation. Experiments were repeated twice with similar results (*** = P < 0.001, one-way ANOVA). (C) As a measure of *Verticillium* colonization, stem sections collected at 21 dpi were placed on agar plates allowing the fungus to grow out of the sections. Pictures were taken after 5 days of incubation.

underneath the cotyledon of five *Verticillium*-inoculated plants per line were harvested, surface sterilized, sliced and placed on PDA plates. Whereas the fungus could only be recovered from some of the stem sections of Moneymaker plants, *Verticillium* grew out from all stem sections of *ΔspAvr2* and *ΔspAvr2^{R45H}* plants (Figure 1C). Similar results were obtained in a second experiment. Overall, these data show that the *ΔspAvr2* and *ΔspAvr2^{R45H}* plants are hyper-susceptible towards *V. dahliae* as depicted by their enhanced fungal colonization and increased disease symptoms.

Avr2 increases susceptibility of tomato to *Botrytis cinerea*

Botrytis cinerea is a necrotrophic plant pathogenic fungus that can infect many plant species. The infection process includes penetration of the host tissue and killing of the host cells, followed by lesion expansion, tissue maceration and sporulation. When cultivated in the greenhouse we often observed *ΔspAvr2* plants to develop spontaneously grey mould disease symptoms on fruits and on wounds after pruning of side shoots. White mycelium emerging at infected areas is a representative symptom of disease caused by *B. cinerea* (Figure 2A). To more systematically test whether Avr2 increases susceptibility to *B. cinerea*, detached leafs of five-week-old tomato plants were inoculated with a droplet of a conidial suspension of strain B05.10 (Figure 2B). In

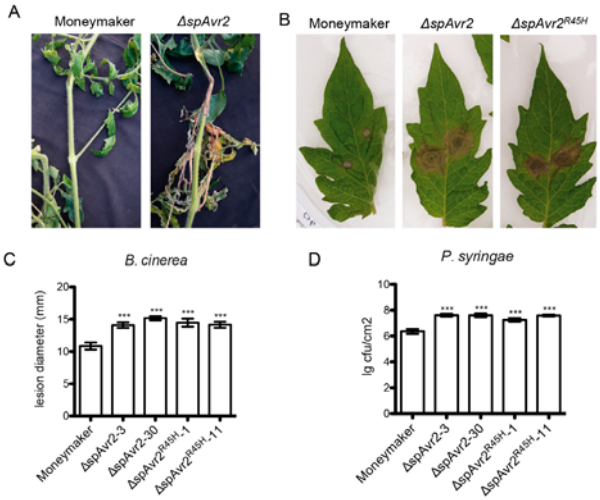

Figure 2. *ΔspAvr2* and *ΔspAvr2^{R45H}* transgenic tomato plants show enhanced susceptibility to *B. cinerea* and *P. syringae*. (A) Representative example of a *B. cinerea* infected *ΔspAvr2* transgenic tomato plant compared to wild-type Moneymaker tomato plants grown in the same compartment. (B) Disease symptoms of *B. cinerea* on tomato leaves. (C) Lesion development of *B. cinerea* on tomato leaves was evaluated at 3 dpi by determining the average lesion diameter on ten leaves from three plants each. (D) Bacterial growth assays on tomato plants inoculated with *P. syringae* by syringe infiltration. Bacterial populations were measured at 3 dpi. Error bar represents means standard deviation. Experiments were repeated at least twice with similar results (*** = P < 0.001, one-way ANOVA).

ΔspAvr2 and *ΔspAvr2*^{R45H} tomato leaves, *B. cinerea* produced significant larger lesions (15mm in diameter) than in wild-type Moneymaker leaves (10mm in diameter) at 3 days post inoculation (dpi) (Figure 2C). These data show that Avr2 enhances susceptibility of tomato to infection with *B. cinerea*.

Avr2 enhances susceptibility of tomato to *Pseudomonas syringae*

To test whether the virulence-promoting activity of Avr2 extends to pathogens other than the fungal pathogens tested, notably *Fo*, *V. dahliae* and *B. cinerea*, its ability to enhance virulence to a bacterial pathogen was assessed. Thereto susceptibility to infection with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) was determined. To monitor progress of disease development four-week-old tomato plants were syringe infiltrated with *Pst* and leaf discs were collected from the infiltrated areas at 3 dpi. A significant increase in bacterial growth was observed (>1 lg cfu/cm^2) in the *ΔspAvr2* and *ΔspAvr2*^{R45H} lines when compared to Moneymaker (Figure 2D). In summary, *ΔspAvr2* and *ΔspAvr2*^{R45H} expression do increase susceptibility of tomato to *Pst*.

ROS production and callose deposition are reduced in *ΔspAvr2* plants

Since expression of *ΔspAvr2* increases the hyper-susceptibility of tomato to a variety of microbial pathogens, we hypothesized that Avr2 might interfere with basal host immune responses. The first layer of plant immunity involves recognition of pathogen-associated molecular patterns (PAMPs) by surface-localized pattern recognition receptors (PRRs) and is referred to as PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). Perception of PAMPs such as bacterial flagellin or its derivative flg22, triggers numerous downstream responses, including production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases, cell wall callose deposition and increased expression of defense related genes (Boller and Felix, 2009). To test whether Avr2 interferes with PTI signaling, we compared the flg22-induced ROS bursts in Moneymaker and two independent *ΔspAvr2* lines using a luminol/peroxidase-based assay. Leaf discs of four-week-old tomato plants were overnight floated on water and than placed carefully in a 96-well plate with 100ul luminol mix containing either flg22 or water. Tomato leave discs incubated with water did not elicit a significant detectable ROS burst (Figure 3A). However, flg22 treatment of Moneymaker led to a burst in ROS formation with a peak of ±150 RLUs at 16 minutes. Compared to the wild-type Moneymaker both *ΔspAvr2* lines showed a severely reduced ROS accumulation as only 50 RLUs were emitted at this time point (Figure 3A). These data show that the flg22-trigged ROS burst is suppressed in *ΔspAvr2* plants, suggesting that PTI signaling is compromised in these plants.

Avr2 represses seedlings growth inhibition induced by flg22 treatment

To further determine whether Avr2 possesses PTI-inhibitory activity, seedling-growth-inhibition induced by flg22 treatment was monitored in *ΔspAvr2* transgenic tomato as described (Pfund et al., 2004). Fresh weight and root length were recorded ten days after transferring the plants to liquid media. Significant growth inhibition was observed in Moneymaker upon flg22 treatment: both plant fresh weight and root length were reduced. Although growth was also inhibited in *ΔspAvr2* plants following flg22 treatment, the reduction was significantly less than that of wild-type plants (Figure 3B and 3C). Additionally, we tested the flg22 treated *ΔspAvr2* plants for callose deposition by staining the cotyledons with aniline blue and determining the number of UV-fluorescent deposits using a fluorescence microscope (see Materials and Methods). The amount of callose deposition was severely reduced in *ΔspAvr2* plants when compared to Moneymaker following flg22 treatment (Figure 3D and 3E). The observed attenuation of ROS production, reduction in growth inhibition and decreased callose deposition indicate that Avr2 is capable of inhibiting the PTI responses.

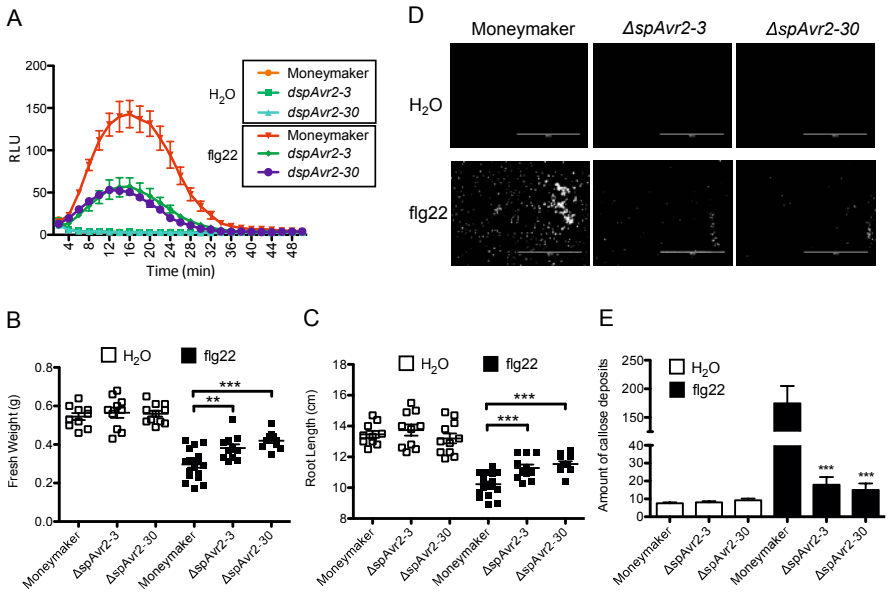

Figure 3. Physiological changes upon pathogen-associated molecular pattern (PAMP) treatment in tomato plants. (A) Flg22 induced oxidative burst in wild-type Moneymaker plants and *ΔspAvr2* plants was measured over a period of 50 minutes. (B) Fresh weight and (C) root length inhibition of tomato plants in liquid Murashige-Skoog media containing either H_2O or flg22 (100nM) was analyzed 7 dpi. (D) Microscopic comparison of callose deposits after flg22 infiltration and staining with aniline blue. (E) Total number of callose deposits per field of view is depicted. Error bars represent standard error of ten biological replicates (*** = P < 0.001, ** = P < 0.05, one-way ANOVA).

Site-directed mutagenesis of Avr2 allows uncoupling of its virulence from its avirulence function

Very recently, the crystal structure of Avr2 has been resolved (Di et al., 2017)(Figure 4A). Avr2 forms a β-sandwich fold with two antiparallel β-sheets. The strands are numbered sequentially from β1 to β7 with sheet I composed of strands β1, β7, β4 and β5 and sheet II containing strands β2, β3 and β6. Additionally, there is a disulfide bond between Cys40 and Cys130 stabilizing the protein fold. The mutated residues occurring in the avirulence breaking variants of race 3 ($Avr2^{V41M}$, $Avr2^{R45H}$, $Avr2^{R46P}$ and $Avr2^{T50-}$) (Houterman et al., 2009; Chellappan et al., 2016) cluster on a surface-exposed loop region (Figure 4B). A homology search through DaliLite identified remote homology with ToxA, an effector from the wheat pathogen *Pyrenophora tritici-repentis* (Manning et al., 2008). However, the conserved Arg-Gly-Asp (RGD)-motif of ToxA, which is required for entry into host plant cells by a proposed interaction with integrin-like receptor proteins (Manning et al., 2008), is not present in Avr2. Avr2 also has structural homology with three other proteins, notably human E3 ubiquitin protein ligase 1 (SIAH1), the tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) (4z8m) and with the MATH- (meprin and TRAF-C homology) domain of Speckle-type POZ (SPOP) protein (3hq1) (Di et al., 2017). TRAF6 interacts with the mitochondrial antiviral signalling protein (MAVS), which oligomerizes in the presence of viral RNA, to activate antiviral immunity (Seth et al., 2005; Shi et al., 2015). SIAH1 and SPOP both exert E3 ubiquitin ligase activity. SPOP contains besides its MATH domain, which recruits the substrate, a BTB domain (Broad Complex, Tramtrack, and Bric-a-Brac) that interacts with E3 Cullin ligases-3 (Cul3) to promote ubiquitination of bound substrates (Zhuang et al., 2009; Xie, 2013). Based on structural homology with these three proteins, site-directed mutagenesis of Avr2 was performed. Residues were

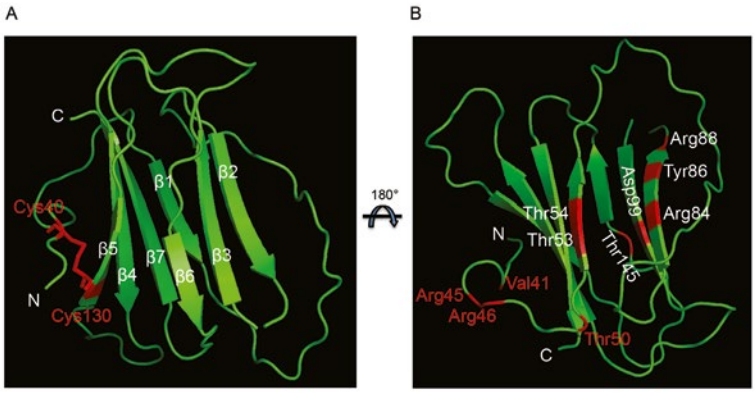

Figure 4. The crystal structure of Avr2 (Di et al., 2017). (A). Avr2 contains a β-sandwich fold with two antiparallel β-sheets. The strands are numbered sequentially from β1 to β7 with sheet I composed of strands β1, β7, β4 and β5 and sheet II containing strands β2, β3 and β6. The disulfide bond is indicated in red. (B). The structure of Avr2 is flipped vertically. The position of the various mutations in Avr2 are highlighted in red in the structure.

selected that are important for the function of these structurally related proteins and their interaction with either substrates or cofactors. Based on SIAH1 (SINA domain) and its interaction with USP19 (ubiquitin specific peptidase), Avr2 mutants were generated in which the threonine residues at either position 53 or 54 (the methionine residue encoded by the first triplet of the *Avr2* open reading frame is numbered 1) were replaced by an arginine ($Avr2^{T53R}$ and $Avr2^{T54R}$), respectively. Both threonines are located on the same face of the protein as the protruding loop harboring the residues ($Avr2^{V41M}$, $Avr2^{R45H}$, $Avr2^{R46P}$ and $Avr2^{T50-}$) that are essential for I-2 mediated recognition (Figure 4B). Based on structures of the MATH domain and its BTB substrate, and of the TRAF6 protein and its interaction with MAVS protein, mutants $Avr2^{T145E}$, $Avr2^{T145K}$, $Avr2^{D99E}$, $Avr2^{D99A}$, $Avr2^{R88A}$, $Avr2^{T86A}$ and $Avr2^{R84A}$ were generated (Figure 4B). The mutants are designed to either replace non-charged residues for charged ones ($Avr2^{T145E}$, $Avr2^{T145K}$, $Avr2^{D99E}$, $Avr2^{T53R}$ and $Avr2^{T54R}$), or *vice versa* ($Avr2^{D99A}$, $Avr2^{R88A}$, $Avr2^{T86A}$ and $Avr2^{R84A}$) in an attempt to interrupt a potential interacting site. These mutations all map at the opposite face of the protein at which the epitope is located that is crucial for Avr2 perception by I-2. Overall, nine amino acid residues in ΔspAvr2 were targeted for mutagenesis based on their synonymous position in the templates, in which these locations are critical for ligand binding.

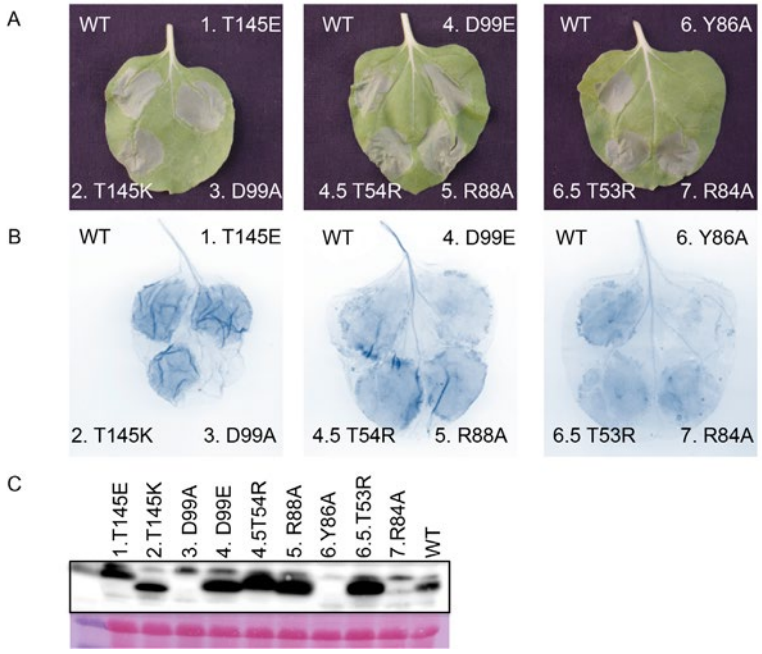

Figure 5. Assessment of I-2-mediated HR in *N. benthamiana* following co-expression of *I-2* with *Avr2* mutants. (A) A transient expression assay in *N. benthamiana* leaves using *A. tumefaciens* co-expressing the *Avr2* mutants with the *I-2* gene. (B) Cell death is visualized by trypan blue staining of the infiltrated leaves. (C) Western blot shows protein accumulation the various Avr2 mutants.

First we assessed whether the mutations affect the ability of the Avr2 mutant to be recognized by I-2. Thereto the various mutants were co-expressed with *I-2* in *Nicotiana benthamiana*, a system that nicely recapitulates the gene-for-gene relation between *I-2* and *Avr2* (Houterman et al., 2009). Wild-type Avr2, but not the three race 3 variants ($Avr2^{V41M}$, $Avr2^{R45H}$ and $Avr2^{R46P}$) triggers an HR in this system (Houterman et al., 2009). As shown in Figures 5A and 5B, wild-type ΔspAvr2 elicits a clear I-2-dependent cell death response and serves as positive control in these experiments. Two of the nine ΔspAvr2 mutants tested ($Avr2^{D99A}$ and $Avr2^{Y86A}$) failed to trigger an HR, whereas the others retained avirulence activity. To examine whether the mutant proteins still accumulate in the variants showing compromised avirulence, a western blot analysis was performed. Avr2 specific antibodies showed equal accumulation of wild-type protein and the mutants $Avr2^{T145E}$, $Avr2^{T145K}$ and $Avr2^{R84A}$, following *Agrobacterium*-mediated expression (Figure 5C). Notably, for $Avr2^{D99E}$, $Avr2^{T54R}$, $Avr2^{R88A}$ and $Avr2^{T53R}$ protein levels appeared to be slightly higher than that of the wild-type effector, suggesting the mutations might stabilize the protein. The $Avr2^{D99A}$ and $Avr2^{Y86A}$ variants did not accumulate to detectable levels, which is consistent with their inability to trigger I-2 activity, suggesting a structural role for these residues in protein stability.

Tomato monocultures selected Avr2 variants that retain virulence, but lost avirulence (Houterman et al., 2009). To determine whether the selected mutants lost their virulence function, their ability to suppress an flg22-induced ROS burst was monitored. Thereto the *Avr2* variants were transiently expressed in *N. benthamiana* leaves. Subsequently, the infiltrated leaves were treated with the flg22 elicitor and generation of ROS was monitored. As shown in Figure 6, *N. benthamiana* leaf discs transiently expressing *GUS* showed a burst in ROS formation following flg22 treatment, which shows that the presence of *A. tumefaciens* did not compromise the ability of the plant to respond to this elicitor. Whereas the presence of wild-type *Avr2* severely reduced ROS accumulation as

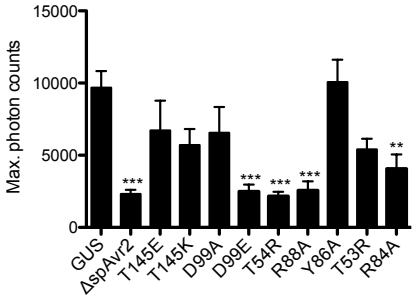

Figure 6. Reactive oxygen species (ROS) suppression by wild-type Avr2 and the derived mutants. The leaves of *N. benthemiana* transiently expressing *Avr2* and its variants after agro-infiltration were treated with the flg22 elicitor and generation of ROS was monitored. Some Avr2 mutants lost their capacity to repress ROS production after flg22 treatment as compared to wild-type Avr2, whereas some Avr2 mutants that retain the capability to repress ROS.

Table1. The summary of virulence and avirulence activity in each Avr2 variants is shown.

Mutation list	HR	Western blot	Suppress ROS
Thr145Glu	+	+	-
Thr145Lys	+	+	-
Asp99Ala	-	-	-
Asp99Glu	+	+	+
Thr54Arg	+	+	+
Arg88Ala	+	+	+
Tyr86Ala	-	-	-
Thr53Arg	+	+	-
Arg84Ala	+	+	+
Wild-type Avr2	+	+	+

compared to the *GUS* control validating the use of this transient system to assess the PTI-suppressing activity of the Avr2 mutants. These observations are consistent with the earlier findings made in tomato (Figure 3A), validating the use of this transient system to assess the PTI-suppressing activity of the Avr2 effector. In line with the western blot analysis, the unstable Avr2^{R99A} and Avr2^{Y86A} mutants did not suppress ROS formation following flg22 treatment. Mutants Avr2^{D99E}, Avr2^{T54R} and Avr2^{R88A}, that retained their avirulence function, could still repress flg22-induced ROS burst and hence resemble the activity of the wild-type protein. In mutants Avr2$^{T145K/E}$ and Avr2^{T53R}, however, the virulence activity was uncoupled from the avirulence function. The virulence and avirulence activity of each Avr2 variants are summarized in table 1.

Discussion

The hyper-susceptibility of transgenic *ΔspAvr2* and *ΔspAvr2^{R45H}* plants to *V. dahliae*, *P. syringae* and *B. cinerea* shows that Avr2 targets a defense component that is important to restrict various plant pathogens. Based on the diversity of the pathogens affected, and their distinct PAMP profiles, it is unlikely that Avr2 targets a specific PRR receptor as PRRs typically have a confined PAMP recognition spectrum (Thomma et al., 2011). ETI and PTI share downstream signaling components (Dodds and Rathjen, 2010), but ETI signaling induced by Rx, Cf4, Pto or I-2 was not compromised in the presence of Avr2 (Gawehns et al., 2014) implying that Avr2 targets an early component specific in PTI signaling. This hypothesis is in line with our observation that both early (ROS production) and late PTI responses (callose deposition and growth inhibition) were impaired in *Avr2* transgenic tomato (Figure 3). Together these data suggest that the Avr2 virulence target acts early in PTI signaling, either being a component of a PRR complex itself, or just acting downstream.

The identity of the Avr2 host-target is unknown, but possible candidates are positive regulators of PRR complexes such as the PRR co-receptor BAK1 (Chinchilla et al., 2007) or kinases such as BIK1 and BSK1 (Veronese et al., 2006; Shi et al., 2013). Alternatively,

like *P. syringae* effector HopM1 Avr2 might target the trafficking process by which plant cells regulate a proper PRR localization at their plasma membrane. HopM1 impairs both early PAMP-triggered responses, like the oxidative burst and stomatal immunity, and late immune responses through targeting the vesicle trafficking-related AtMIN7 for degradation (Lozano-Duran et al., 2014). Finally, Avr2 might compromise PRR functioning by interfering with PRR biogenesis. A strict endoplasmic reticulum (ER)-quality control mechanism ensures that only properly folded and functional transmembrane immune receptors are secreted to the plasma membrane (Lozano-Duran et al., 2014). Interference with any of the steps involved in the quality control process by Avr2 might affect this process resulting in non-functional PTI receptor complexes. Although currently none of the above options can be excluded, we strongly favor the first one. If Avr2 would interfere with fundamental processes such as the biosynthesis, quality control and/or translocation of trans-membrane receptors from the ER to the plasma membrane, strong developmental defects, which were not observed (Figure 1B and 2A), would be expected in the *Avr2* transgenic lines as similar receptors are involved in control of growth and development (Hecht et al., 2001).

It has been shown that *Fol* race 3 isolates carry single amino acid substitutions in Avr2 ($Avr2^{V41M}$, $Avr2^{R45H}$, $Avr2^{R46P}$ and $Avr2^{T50-}$) that abolish I-2-mediated recognition, but that do not affect its virulence function (Houterman et al., 2009; Chellappan et al., 2016). Unlike the loss of *Avr1* in race 2 and 3 isolates, showing its dispensability, *Avr2* or its variants are always retained in *Fol*, which is consistent with its role as important virulence factor for the fungus. The mechanism that applies to effector recognition by the NB-LRR protein I-2 is unknown: recognition could be either direct or indirect. The Avr2 crystal structure revealed that the residues in $Avr2^{V41M}$, $Avr2^{R45H}$, $Avr2^{R46P}$ and $Avr2^{T50-}$ that allow the effector to evade I-2 recognition cluster at a loop forming a distinct epitope (Figure 4B). The observation that these Avr2 variants are unaffected in their virulence activity, but lost their avirulence function, shows that these activities can be uncoupled. In agreement, our targeted mutagenesis identified two threonine residues ($Avr2^{T53}$ and $Avr2^{T145}$) in Avr2 that are essential for virulence, but not for avirulence. The mutations in variants $Avr2^{D99A}$ and $Avr2^{Y86A}$ also disrupt recognition, but they render the protein unstable. So, virulence and avirulence function are distinct events that can be uncoupled and the residues involved in these activities are not overlapping. Together this implies that I-2 mediated recognition is a direct event and does not involve the changes that Avr2 exerts on a putative virulence target in the cell. Besides direct recognition of Avr2 alone, recognition by I-2 might be based on the proximity of two epitopes, one present on the loop region in Avr2 and the other present on an interacting host target.

The structural homology search identified ubiquitin protein ligases as potential structural homologs for Avr2. Ubiquitination, a highly conserved eukaryote-specific

post-translational protein modification, plays a key role in cell-cycle regulation, DNA repair, cell growth and immune responses (Weissman, 2001). Our functional profiling identified residues T53 and T145 in Avr2 as critical for virulence function as Avr2^{T53R} and Avr2^{T145} failed to suppress PTI. The Avr2^{T53R} and Avr2^{T145} variants are still recognized by I-2 implying a correct fold of the mutant proteins. The synonymous residues T53 and T145 are mutated based on the different templates SIAH1 and TRAF6, respectively. It is therefore tempting to speculate that Avr2 either itself acts as an ubiquitin ligase or interferes with the activity of plant ubiquitin ligases by competing for substrate binding. If *Avr2* encodes a ligase it might promote ubiquitination-mediated degradation of a specific target protein that is a positive regulator of PTI signaling, such as BAK1 as discussed before. When competing for a substrate it might prevent the degradation of a negative regulator by preventing ubiquitinylation by the host ligase. Only few studies have been published that address molecular mechanisms underlying perturbation of plant ubiquitin systems by microbial effectors. The AVR3a protein from the *Phytophthora infestans*, important for virulence of this oomycete, interacts with and stabilizes the immunity-related U-box E3 ligase protein CMPG1 (Bos et al., 2010). The *M. oryzae* effector, AvrPiz-t, suppresses innate immune responses associated with early perception of the pathogen and enhances susceptibility in rice plants. AvrPiz-t interacts with an E3 ligase called APIP6 and suppresses its activity *in vitro* (Park et al., 2012). Besides effectors directly targeting host E3 ubiquitin-ligase proteins, also E3 ubiquitin-ligase-related domains in effector protein have been reported (Marino et al., 2012). Fen, a homologue of immunity-related kinase Pto, is ubiquitinated by the AvrPtoB effector from *Pseudomonas syringae*.

Resolving the molecular mechanism on how Avr2 compromises plant immunity awaits identification of the Avr2 host target. So far our attempts to identify such a target using yeast two-hybrid screens and pull downs assays have been unsuccessful (Ma et al., 2015). However, the structural conservation of Avr2 with ubiquitin ligases provides new leads to identify its target. Identification of the target and making it insensitive to Avr2 activity might result in a reduced susceptibility to *Fol* infection as well as in a potentiated PTI response preventing other pathogens to cause disease on *Fol* infected plants.

Materials and methods

Plant material and fungal and bacterial strains

Tomato (*Solanum lycopersicum*) cultivar Moneymaker was used. Tomato plants were germinated and grown in soil with 16/8 h light/dark cycles, at 22/16°C day/night and 70% relative humidity in the green house.

The *V. dahliae* race 1 JR2 and *B. cinerea* wild-type strain B05.10 was provided by Bart

P.H.J. Thomma (WUR, Wageningen, The Netherlands) and Jan A. L. Van Kan (WUR, Wageningen, The Netherlands), respectively (Fradin et al., 2009; Zhang and Van Kan, 2013). Pathogenic bacterial strain *Pst* DC3000 was used in this study (Whalen et al., 1991). Deletion of *Avr2* in *Fol007* background (*FolΔAvr2*) has been described previously (Houterman et al., 2009).

Construction of binary vectors

ΔspAvr2^{R45H} was amplified with primers FP2525 and FP2274 using CTAPi::*ΔspAvr2*^{R45H} as a template (Houterman et al., 2009). The obtained products were cloned into the vector SLDB3104 (Tameling et al., 2010) between the *Xba*I and *Bam*HI restriction sites to generate SLDB3104::*ΔspAvr2*^{R45H}. SLDB3104::*ΔspAvr2* has been described before (Ma et al., 2013). In the resulting plasmid Avr2 was fused to a C-terminal HA and streptavidin-binding peptide (SBP) tag. All PCR primers listed in table 2 were purchased from MWG (http://www.mwg-biotech.com), and sequences of all plasmids were confirmed by sequence analysis. Avr2 was cloned behind the cauliflower mosaic virus 35S promoter for constitutive expression. The resulting vector was introduced by electroporation into LBA4404 (Hoekema et al., 1983) for tomato transformation.

Plant transformation

Moneymaker was transformed with the construct described above using *Agrobacterium*-mediated transformation in tomato as described before (Cortina and Culianez-Macia, 2004). First-generation transformants of *ΔspAvr2* and *ΔspAvr2*^{R45H} were selected on 1/2 Murashige and Skoog (MS) medium containing kanamycin (40mg/L). To select *ΔspAvr2* transgenic lines, T1 progeny were analyzed by scoring the ratio of kanamycin-resistant to kanamycin-sensitive seedlings. Subsequently the kanamycin-resistant plants were transferred to soil for self-fertilization. The homozygous single insertion lines in the 26 independent T2 generations were selected according to segregation analyses. 25 plants of each independent T2 lines were checked by PCR with primer pairs FP962 and FP963 detecting the approximately 273-bp fragment of the *Avr2* gene. Only two lines harbored the transgene *Avr2* in all tested 26 lines (*ΔspAvr2-3* and *ΔspAvr-30*). Homozygous *ΔspAvr2*^{R45H} lines were screened by the same procedure. In the end, two of 17 *ΔspAvr2*^{R45H} plants (*ΔspAvr2*^{R45H}*-1* and *ΔspAvr2*^{R45H}*-11*) were kept for further study.

V. dahliae inoculation assay

Ten-day-old tomato plants were carefully uprooted from the soil and the roots were placed in a race 1 *V. dahliae* JR2 inoculum (10^6 conidia/ml) for 5 min (Fradin et al., 2009). Thereafter, the plants were transferred to fresh soil. After two weeks disease symptom were scored by measuring the canopy surface of the plants. Fungal colonization in planta was assessed at 21 dpi following inoculation with *V. dahliae*. To this end, stem sections taken just above cotyledon of five representative plants were harvested and

surface-sterilized by sequential treatment for 15 minutes with 70% ethanol and 10% sodium hypochlorite. After three times 5 min washing in sterile water, stem disks of five individual plants about 4 mm thick were cut and placed on potato dextrose agar (PDA) supplemented with 34ug/ml chloramphenicol. The plates were incubated at room temperature and pictures of fungal outgrowth were taken after 10 days.

B. cinerea inoculation assay

Wild-type *B. cinerea* strain B05.10 was grown on Malt Extract Agar (Oxoid, Basingstoke, UK; 50g/l) in the dark at 20 °C for 3-4 days. The plates were placed for one night under near-UV light (350–400 nm) and were subsequently returned to darkness to promote sporulation. Spores were harvested 4-7 days later in 20 mL of water, and the suspension was filtered over glass wool to remove mycelium fragments. The spore suspension was centrifuged at 2000g for 5 minutes. The supernatant was discarded and the spores in the pellet were resuspended at the desired density. Droplets of a suspension of conidia of *B. cinerea* (2ul, 5×10^6 conidia/ml in potato dextrose broth, 1.2g/l) were inoculated on the leaves of six-week-old tomato plants (Zhang and Van Kan, 2013). The pictures were captured by canon camera and lesion diameters were measured by Image J software at 3 dpi.

Pst DC3000 inoculation assay

Pst DC3000 was grown at 28°C on King's B liquid medium (KB) containing 40ug/ml rifampicin (King et al. 1954) for 48 h. Prior to inoculation, bacteria were collected by centrifugation (5 min, 1000 g) and the pellet was resuspended in 1 ml of 10mM $MgSO_4$ to an Optical Density (OD)600 of 0.0005. The bacteria were syringe infiltrated into the leaves of four-week-old tomato. At 3 dpi, leaf discs (diameter = 6 mm) were collected and the bacteria were extracted in 10 mM $MgSO_4$. Serial dilutions (typically 10^{-3}, 10^{-4}, and 10^{-5}) of the bacteria suspension were plated on King's B plates containing 40ug/ml rifampicin (Dong et al., 1991). Colonies were counted after two days of incubation at 28°C. The assay was repeated twice with similar results.

Flg22 induced tomato seedlings growth inhibition assay

Flg22 induced seedling growth inhibition assays (Gomez-Gomez et al., 1999) were performed as described (Pfund et al., 2004). Tomato seeds were sterilized for 2 min in 70% ethanol, then 10 min in 3% thin bleach, and subsequently washed three times in sterile H_2O. Seeds pre-germinated on 1% water agar for 48 h were transferred to 250ml glass flask containing 200 ml of 1/2 MS and 1% (w/v) sucrose liquid media supplemented with either 100nM flg22 or water (ten seedlings per flask). The seedlings were incubated at 21°C with 12 hours of light and 12 hours of darkness while shaking at 100rpm/h. Fresh weight and root length were recorded after 7 days. The assay was repeated twice with similar results.

Callose deposition

Callose was visualized as described (Gomez-Gomez et al., 1999). Briefly, the leaf discs from the flg22 growth inhibition assay were dissected and cleared with 70% ethanol for 1 hour, then 100% ethanol overnight until all chlorophyll was removed. Cleared leaves were rehydrated sequentially for 30 minutes in 50% ethanol. Callose was stained in a 0.01% aniline blue solution in 150mM K_2HPO4 at pH 9.5 for 2 h. The leaf discs were mounted in a solution of 70% glycerol and examined by UV fluorescence under fluorescent microscope (EVOS). Callose foci within the frame of a single image (magnification ×4) were counted by image J software. Eight adjacent fields of view along the length of each leaf were analyzed and the values are the average of four independent plants. The assay was repeated twice with similar results.

Oxidative burst assay

ROS measurement was performed using a luminol/peroxidase-based assay (Felix et al., 1999). Leaf discs of four-week-old tomato plants (diameter = 6 mm) or leaf discs of *N. benthemiana* were dissected with a puncture and floated in sterile water overnight. The single leaf disc was then transferred to each well of a 96-well plate containing 100ul H_2O supplied with 250nM luminol and 1ug/ml horseradish peroxidase (HRP). Four individual plants using three leaf discs per plant were treated. Luminescence was recorded over a 50-min period using a Magellan F50 (TECAN) plate reader after treatment with either 100nM flg22 or H_2O and then displayed as the sum of photon counts over this period. This assay was repeated twice with similar result.

Site mutagenesis of Avr2

The side directed mutants were generated using quick-change mutagenesis (Zheng et al., 2004). As template for mutagenesis pDONR207::*ΔspAvr2* was used (Houterman et al., 2009). The PCR product was treated with *Dpn*I and transformed to E.coli DH5α competent cells. All PCR primers were purchased from MWG (http://www.mwg-biotech.com), and sequences of all plasmids were confirmed by sequence analysis. The obtained plasmids were introduced into the binary cTAPi vector (Rohila et al., 2004) by the gateway protocol.

Agrobacterium-mediated transient transformation of *N. benthamiana*

A. tumefaciens strain GV3101 was transformed with binary constructs as described previously (Ma et al., 2012). Briefly, the agrobacteria were grown to an absorbance of 0.8 at 600 nm in LB-mannitol medium (10g/l tryptone, 5g/l yeast extract, 2.5g/l NaCl, 10g/l mannitol) supplemented with 20um acetosyringone and 10mM MES pH 5.6. Cells were pelleted by centrifugation at 4000g at 20°C for 20 min and then resuspended in infiltration medium (1× MS salts, 10mM MES pH 5.6, 2% w/v sucrose, 200um acetosyringone). Infiltration was done in *N. benthamiana* leaves at an absorbance of 0.2

(for I-2 constructs) (van Ooijen et al., 2008) or 0.5 (for Avr2 constructs) of 4–5-weeks-old plants.

Trypan blue staining
Leaves were boiled for 5 min in a 1:1 mixture of 96% ethanol and staining solution (100ml lactic acid, 100ml phenol, 100ml glycerol, 100ml H_2O and 100mg Trypan bule). Then the leaves were destained in 2.5g/ml chloral hydrate in water (Ma et al., 2012).

Protein extraction and western blotting
Protein extraction was done as described previously (Ma et al., 2015). To verify presence of Avr2 in transgenic tomato plants and in *N. benthamiana*, leaves were harvested and snap-frozen in liquid nitrogen. After grinding the tissue with a mortar and a pestle, the powder was allowed to thaw in 2 ml protein extraction buffer per gram of tissue [25mM Tris pH 8, 1mM EDTA, 150mM NaCl, 5mM DTT, 0.1% NP-40, 1 Roche complete protease inhibitor cocktail (http://www.roche.com) and 2% PVPP]. Extracts were centrifuged at 12,000 g, 4°C for 10 min, and the supernatant was passed over four layers of miracloth to obtain a "total" protein lysate. 20ul samples were mixed with Laemmli sample buffer and were run on 13% SDS–PAGE gels and blotted on PVDF membranes using semi-dry blotting. Skimmed milk powder (5%) was used as a blocking agent. The membranes were subjected to immunoblotting using either anti-HA peroxidase at a dilution of 1:3000 (clone 3F10; Roche) or anti-Avr2 antibody (1:5,000 diluted) (Ma et al., 2015). The secondary antibody goat-anti-rat (P31470, Pierce) was used at a 1:5000 dilution. The luminescent signal was visualized by ECL using BioMax MR film.

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Table 2. Primers used in this study

Name	Target gene	Sequences (5'-3')
FP2525	*ΔspAvr2*^{R45H}-F	CGCTCTAGAATGCCTGTGGAAGATGCCGAT
FP2274	*ΔspAvr2*^{R45H}-R	GCGGGATCCTCCATCCTCTGAGATAGTAAG
FP962	*Avr2*-F	TGAGCGGGCTGGCAATTC
FP963	*Avr2*-R	CAATCCTCTGAGATAGTAAG
FP6915	*Avr2*^{T145E}-F	GCTCTCGAGGTCCAGCCGAGATCAGTTGGGATGCCGA
FP6916	*Avr2*^{T145E}-R	TCGGCATCCCAACTGATCTCGGCTGGACCTCGAGAGC
FP6917	*Avr2*^{T145K}-F	GCATCCCAACTGATTTTGGCTGGACCTCGAG
FP6918	*Avr2*^{T145K}-R	CTCGAGGTCCAGCCAAAATCAGTTGGGATGC
FP6919	*Avr2*^{D99A}-F	GGGGGGCGACAGCAATGACAGTGCGGAG
FP6920	*Avr2*^{D99A}-R	CTCCGCACTGTCATTGCTGTCGCCCCCC
FP6921	*Avr2*^{D99E}-F	GGGGGGGCGACCTCAATGACAGTGCGGA
FP6922	*Avr2*^{D99E}-R	TCCGCACTGTCATTGAGGTCGCCCCCCC
FP6923	*Avr2*^{T54R}-F	GGTGCTGAAGCTCCTAGTAAATGAAGTAGAAGACGTGC
FP6924	*Avr2*^{T54R}-R	GCACGTCTTCTACTTCATTTACTAGGAGCTTCAGCACC
FP6925	*Avr2*^{R88A}-F	CTCCAACGCGACTTGCTTCGTAAATGCGGTGATTCAGTCC
FP6926	*Avr2*^{R88A}-R	GGACTGAATCACCGCATTTACGAAGCAAGTCGCGTTGGAG
FP6927	*Avr2*^{Y86A}-F	GCGACTTCGTTCGGCAATGCGGTGATTCAGTCCCGAAT
FP6928	*Avr2*^{Y86A}-R	ATTCGGGACTGAATCACCGCATTGCCGAACGAAGTCGC
FP6929	*Avr2*^{T53R}-F	TGCTGAAGCTCGTCCTAAATGAAGTAGAAGACGTGCGGCG
FP6930	*Avr2*^{T53R}-R	CGCCGCACGTCTTCTACTTCATTTAGGACGAGCTTCAGCA
FP6931	*Avr2*^{R84A}-F	GCGACTTCGTTCGTAAATGGCGTGATTCAGTCCCGAATTG
FP6932	*Avr2*^{R84A}-R	CAATTCGGGACTGAATCACGCCATTTACGAACGAAGTCGC

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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

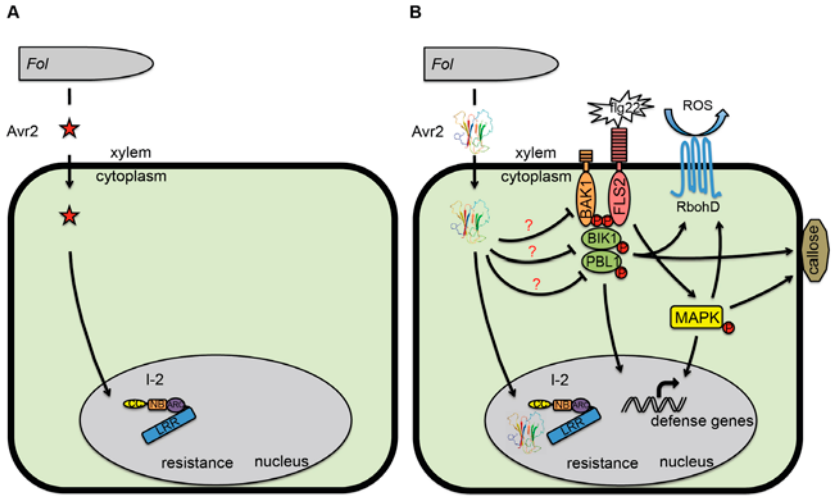

Figure 1. An updated model for the molecular functions of Avr2 in tomato. (A) *Fol* secretes Avr2 into the xylem vessels and apoplastic spaces of tomato plants. Avr2 acts inside the plant cell to exert its virulence function. In a resistant plant, *I-2* containing plant immune responses are triggered 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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Summary

Fusarium oxysporum (*Fo*) is one of the top ten fungal pathogens worldwide (Dean et al., 2012). It causes vascular wilt disease in over 120 different plant species, including the economically important crops banana, cotton, melon, tomato and the weed Arabidopsis (Michielse and Rep, 2009). Individual isolates of *Fo* usually cause disease on a narrow range of host plant species or on a single species only. Based on this observation isolates of this plant pathogenic fungus have been classified into *formae speciales*, each *formae specialis* (f.sp.) is named after the specific plant species it infects (Michielse and Rep, 2009). **Chapter 2** provides an overview on the role of the major phytohormones in the interaction between various *formae speciales* of *Fo* and their specific hosts. A model is presented in which Salicylic Acid (SA) signaling reduces susceptibility of the host to *Fo*. The effects of Jasmonic Acid (JA), Ethylene (ET), Abscisic Acid (ABA) and auxin, however, are more complex and differ between pathosystems. We speculate that the latter phytohormone-signaling pathways may be hijacked by *Fo* for host manipulation. Ultimately, the mechanisms are discussed on how plant hormones and *Fo* effectors balance the plant-fungus interaction from beneficial to pathogenic and *vice versa*.

The remainder of the thesis is focused on *Fo* f.sp. *lycopersici* (*Fol*), a soil-borne and xylem-colonizing pathogen, that causes vascular wilt disease in tomato (*Solanum lycopersicum*) only. In **Chapter 3** the role of the major phytohormone signaling pathways in tomato towards susceptibility to *Fol* is studied. Bioassays of SA, ET and JA deficient mutants of tomato were performed with wild-type *Fol* or the less pathogenic *FolΔAvr2* strain. It was found that impaired SA signaling enhances *Fol* disease symptoms, whereas ET is responsible for promoting disease development. JA might be manipulated by the *Fol* effector protein Avr2 and was found to play a role in the ability of the fungus to colonize tomato. Finally, a model for the role of SA, ET and JA signaling in susceptibility of tomato to *Fol* is proposed and compared to that in Arabidopsis. The function of the ET, SA and JA in susceptibility to *Fo* seems broadly conserved between *A. thaliana* and tomato.

Upon colonization *Fol* secretes over 14 proteins called Six (Secreted in xylem) proteins in the xylem sap of infected tomato plants (Rep et al., 2005; Houterman et al., 2007). One of these, notably Six3 (Avr2), is not only required for full virulence, but functions as an Avirulence (Avr) protein as well: it triggers *Fol* resistance in the host that is mediated by the Avr2-matching Resistance (R) protein I-2 (Houterman et al., 2009). To get insights into its virulence function, *Avr2* was heterologously expressed in tomato plants in **Chapter 4**. Pathogenicity of an *Avr2* knockout *Fol* (*FolΔAvr2*) strain was fully restored in transgenic tomato lines that produce either secreted (*Avr2*) or cytosolic *Avr2* (*ΔspAvr2*) protein. The latter suggests that although Avr2 was isolated from the xylem

sap it exerts its virulence functions inside the host cell. This implies that secreted Avr2 is taken up from the extracellular spaces. In addition, evidence has been obtained that Avr2 uptake is not a host autonomous process, and that both *Fol* and *Agrobacterium*, but not *Verticillium dahliae*, facilitates Avr2 uptake by tomato cells from extracellular spaces. Based on these results, we propose a novel strategy (Takken et al., 2016) to confer disease resistance in plants.

In **Chapter 5** transgenic tomato plants producing Avr2 were found to become hyper-susceptible towards non-related plant pathogens and have a compromised PAMP triggered-immunity. Suppression of PTI might be the main virulence function of Avr2 and provides insight in the processes targeted by the effector. A structure-guided Avr2 mutagenesis approach was used to determine, in some detail, the regions in the protein involved in its virulence and avirulence function. The results confirmed the idea that these two functions can be uncoupled and are conferred by distinct regions on the protein.

The results described in this thesis are summarized in **Chapter 6**. A model for the role of Avr2 in susceptibility and I-2 mediated resistance is proposed. To further extend the proposed model the Avr2 host target should be identified. Its identification will lead to a better understanding of the function of Avr2 in virulence and possibly in I-2-mediated resistance during *Fol* infection.

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Samenvatting

Fusarium oxysporum (*Fo*) staat in de wereldwijde top-tien van schimmels die in staat zijn planten ziek te maken (Dean et al., 2012). Het pathogeen veroorzaakt verwelkingsziekte in ruim 120 verschillende plantensoorten, waaronder het onkruid Arabidopsis (in het Nederlands: Zandraket) en economische belangrijke gewassen zoals banaan, katoen, meloen en tomaat (Michielse and Rep, 2009). Ondanks het grote aantal *Fo* gastheren kan een individueel isolaat slechts een, of enkele, plantensoorten infecteren. Op basis hiervan wordt de schimmel ingedeeld in zogenoemde *formae speciales*. Elke *forma specialis* (f.sp.) is vernoemd naar de specifieke waardplant waarin deze schimmel ziekte veroorzaakt (Michielse and Rep, 2009).

Hoofdstuk 2 geeft een overzicht van de rol van de belangrijkste plantenhormonen in de interactie tussen de verschillende formae speciales van *Fo* en hun specifieke gastheren. Een model wordt gepresenteerd waarin Salicylzuur (SA) de vatbaarheid van een plant voor verlaagd. De rol van Jasmijnzuur (JA), Ethyleen (ET), abscisinezuur (ABA) en auxine zijn complexer en verschillen voor de verschillende pathosystemen. We speculeren dat de fytohormoon-signaleringsroutes gemanipuleerd worden door de schimmel om de gastheer te kunnen bespelen. Verder bespreken we mogelijke mechanismen hoe planthormonen en *Fo* effectoreiwitten de relatie tussen de plant en schimmel beïnvloeden. De relatie tussen *Fo* en een gastheer plant varieert van ziekmakend tot een wederzijds voordelige.

De rest van het proefschrift richt zich op *Fo* f.sp. *lycopersici* (*Fol*), een in de bodem voorkomende schimmel die de xyleemvaten van tomatenplanten (Solanum lycopersicum) infecteert en daarbij verwelkingsziekte veroorzaakt. **Hoofdstuk 3** richt zich op de betrokkenheid van de eerder genoemde planthormonen bij de gevoeligheid voor *Fol* infectie. Planten met een defect in SA, ET of JA signalering werden geïnoculeerd met wild-type *Fol* of met de minder agressieve *FolΔAvr2* stam. Gevonden werd dat een verstoorde SA signalering de *Fol* ziekte symptomen versterkt, terwijl een verstoorde ET signalering de symptomen juist vermindert. JA signalering wordt mogelijk beïnvloed door het *Fol* effector eiwit Avr2. Daarnaast lijkt dit hormoon betrokken bij het vermogen van de schimmel om de plant te koloniseren. Op basis van de verkregen data wordt een model gepresenteerd met daarin aangegeven de rol van SA, ET en JA signalering in de vatbaarheid van tomaat voor *Fol*. Dit model wordt vergeleken met dat voor Arabidopsis; de rol van ET, SA en JA in gevoeligheid voor *Fo* lijkt grotendeels geconserveerd te zijn tussen beide plantensoorten.

Tijdens kolonisatie van de xyleemvaten van een tomatenplant scheidt de schimmel kleine eiwitten uit in het xyleemsap. Uit het sap van geïnfecteerde planten zijn inmiddels

14 van deze zogenaamde Six (Secreted in xylem) eiwitten geïsoleerd (Rep et al., 2005; Houterman et al., 2007). Een van deze eiwitten, Six3 (Avr2), heeft naast een virulentie functie (dwz het vermogen van de schimmel om de plant ziek te maken) ook een rol in avirulentie. Het Avr2 eiwit induceert onbedoeld een afweerrespons in tomatenplanten die het I-2 resistentie eiwit produceren (Houterman et al., 2009). Om inzicht te krijgen in de virulentie functie van Avr2 werd het eiwit heteroloog tot expressie gebracht in tomatenplanten. Zoals beschreven in **Hoofdstuk 4** kan plant-geproduceerd Avr2 eiwit, zowel wild type Avr2 als de cytosolische ΔspAvr2 variant, de virulentie van een *Avr2* knockout *F. oxysporum* (*FolΔAvr2*) stam volledig complementeren. Dit betekent dat, hoewel Avr2 gevonden wordt in het xyleemsap van de plant, het eiwit zijn functie uitoefent binnen in de cellen van de gastheer. Dit impliceert opname van het eiwit door de plantencel vanuit de extracellulaire ruimtes. Deze opname blijkt geen autonoom proces te zijn en de aanwezigheid van het pathogeen te vereisen. Gevonden werd dat zowel *Fol* als de bacterie *Agrobacterium*, maar niet de schimmel *Verticillium dahliae*, de opname van Avr2 door tomaten cellen kan faciliteren. Op basis van deze ontdekking is een patent ingediend (Takken et al., 2016) waarin een strategie wordt beschreven waarmee mogelijk een brede ziekteresistentie in planten verkregen zou kunnen worden.

In **Hoofdstuk 5** wordt beschreven dat transgene, Avr2 producerende tomatenplanten hyper-vatbaar zijn voor verschillende, niet gerelateerde, plant pathogenen. Daarnaast blijkt in deze Avr2 planten de PAMP geïnduceerde afweerresponse (PTI) verstoord te zijn. Het onderdrukken van deze PTI response lijkt een belangrijke virulentie functie van Avr2 te zijn. Deze ontdekking biedt aanknooppunten om de specifieke processen te identificeren die verstoord worden door Avr2. Op basis van de recent opgehelderde eiwitstructuur is een structuur/functie analyse gedaan en zijn aminozuren geïdentificeerd die belangrijk zijn voor de virulentie en avirulentie functies van het eiwit. Gevonden werd dat beide functies ontkoppeld kunnen worden en op verschillende plaatsen in het eiwit gelokaliseerd zijn.

De resultaten van het proefschrift worden besproken in **hoofdstuk 6**. Hierin wordt een model gepresenteerd voor de rol van Avr2 in vatbaarheid en I-2-gemedieerde resistentie. Om dit model verder uit te bouwen is identificatie nodig van het planteneiwit dat gemanipuleerd wordt door Avr2. Karakterisatie van dit eiwit leidt waarschijnlijk niet alleen tot een beter begrip van de rol van Avr2 in virulentie, maar vergroot ook ons inzicht in de I-2-gemedieerde afweer tijdens *Fol* infectie.

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