Multiple faces of Fusarium oxysporum effector protein Avr2
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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:
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 *Avr2R45H* 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 ΔspAvr2R45H 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 Avr2T145E/K and Avr2T53R 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 loses 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 ($\Delta$spAvr2), effectively complements fungal virulence of a Fol$\Delta$Avr2 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 $\Delta$spAvr2 and $\Delta$spAvr2$^{R45H}$ transgenic tomato plants were inoculated with spores of a race isolate 1 of *V. dahliae* (strain JR2). Besides wild-type Moneymaker, two independent $\Delta$spAvr2-expressing tomato lines ($\Delta$spAvr2-3 and $\Delta$spAvr2-30) and two $\Delta$spAvr2$^{R45H}$ expressing tomato lines ($\Delta$spAvr2$^{R45H}$-1 and $\Delta$spAvr2$^{R45H}$-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 $\Delta$spAvr2 and $\Delta$spAvr2$^{R45H}$ 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. $\Delta$spAvr2 and $\Delta$spAvr2$^{R45H}$ transgenic tomato plants show enhanced susceptibility to *V. dahliae*.](image)

(A) Representative pictures of mock (upper row) and race 1 JR2 (bottom row) inoculated Moneymaker and $\Delta$spAvr2 and $\Delta$spAvr2$^{R45H}$ 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 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<sup>R45H</sup> plants (Figure 1C). Similar results were obtained in a second experiment. Overall, these data show that the ΔspAvr2 and ΔspAvr2<sup>R45H</sup> 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 leaves 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<sup>R45H</sup> transgenic tomato plants show enhanced susceptibility to *B. cinerea* and *P. syringae.*](image)

(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 ΔspAvr2R45H 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/cm2) in the ΔspAvr2 and ΔspAvr2R45H lines when compared to Moneymaker (Figure 2D). In summary, ΔspAvr2 and ΔspAvr2R45H 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-triggered 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 H₂O 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 < 0.05, 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 (Avr2V41M, Avr2R45H, Avr2R46P and Avr2T50-) (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.](image-url)
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$^{D99A}$
and Avr2$^{T53R}$) 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.](image-url)
Chapter 5

Table 1. The summary of virulence and avirulence activity in each Avr2 variants is shown.

<table>
<thead>
<tr>
<th>Mutation list</th>
<th>HR</th>
<th>Western blot</th>
<th>Suppress ROS</th>
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<tbody>
<tr>
<td>Thr145Glu</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
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<td>+</td>
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<td>Thr53Arg</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wild-type Avr2</td>
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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 Avr2R99A and Avr2Y86A mutants did not suppress ROS formation following flg22 treatment. Mutants Avr2D99E, Avr2T54R and Avr2R88A, that retained their avirulence function, could still repress flg22-induced ROS burst and hence resemble the activity of the wild-type protein. In mutants Avr2T145K/E and Avr2T53R, 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 ΔspAvr2R45H 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 (Avr2V41M, Avr2R45H, Avr2R46P and Avr2T50-) 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 Avr2V41M, Avr2R45H, Avr2R46P and Avr2T50- 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 (Avr2T53 and Avr2T145) in Avr2 that are essential for virulence, but not for avirulence. The mutations in variants Avr2D99A and Avr2Y86A 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 greenhouse.

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
\( \Delta sp\text{Avr2}\text{R45H} \) was amplified with primers FP2525 and FP2274 using CTAPi::ΔspAvr2\text{R45H} as a template (Houterman et al., 2009). The obtained products were cloned into the vector SLDB3104 (Tameling et al., 2010) between the XbaI and BamHI restriction sites to generate SLDB3104::ΔspAvr2\text{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 \( \Delta sp\text{Avr2} \) and \( \Delta sp\text{Avr2}\text{R45H} \) were selected on 1/2 Murashige and Skoog (MS) medium containing kanamycin (40mg/L). To select \( \Delta sp\text{Avr2} \) 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 \( \text{Avr2} \) gene. Only two lines harbored the transgene \( \text{Avr2} \) in all tested 26 lines (\( \Delta sp\text{Avr2}-3 \) and \( \Delta sp\text{Avr2}-30 \)). Homozygous \( \Delta sp\text{Avr2}\text{R45H} \) lines were screened by the same procedure. In the end, two of 17 \( \Delta sp\text{Avr2}\text{R45H} \) plants (\( \Delta sp\text{Avr2}\text{R45H}-1 \) and \( \Delta sp\text{Avr2}\text{R45H}-11 \)) were kept for further study.

\textit{V. dahliae} inoculation assay
Ten-day-old tomato plants were carefully uprooted from the soil and the roots were placed in a race 1 \textit{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 \textit{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, 5x10⁶ 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 MgSO₄ 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 MgSO₄. 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 H₂O. 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$_2$HPO$_4$ 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. benthamiana* 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$_2$O 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$_2$O 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 DpnI 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 acetylsyringone 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 (1x MS salts, 10mM MES pH 5.6, 2% w/v sucrose, 200um acetylsyringone). 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$_2$O and 100mg Trypan blue). 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

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Fusarium oxysporum Avr2 compromises PTI in tomato
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