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### Multiple faces of *Fusarium oxysporum* effector protein Avr2

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**Publication date**

2017

**Document Version**

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**Citation for published version (APA):**

Di, X. (2017). *Multiple faces of Fusarium oxysporum effector protein Avr2*. [Thesis, fully internal, Universiteit van Amsterdam].

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## Chapter 5

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

5

**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<sup>R45H</sup> 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  $\Delta spAvr2$  (lacking the signal peptide encoding sequence) or the  $\Delta spAvr2^{R45H}$  variant both fully complemented the virulence defect of *Fol* $\Delta Avr2$ , showing the functionality of the plant-produced effector. Notably,  $\Delta spAvr2$  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  $\Delta spAvr2$  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  $\Delta spAvr2$  plants. Structure-guided mutagenesis allowed uncoupling the virulence from the avirulence function: mutants Avr2<sup>T145E/K</sup> and Avr2<sup>T53R</sup> 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*<sup>R45H</sup> variant in tomato. *Avr2*<sup>R45H</sup> 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  $\Delta spAvr2$  and  $\Delta spAvr2$ <sup>R45H</sup> 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  $\Delta spAvr2$  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.

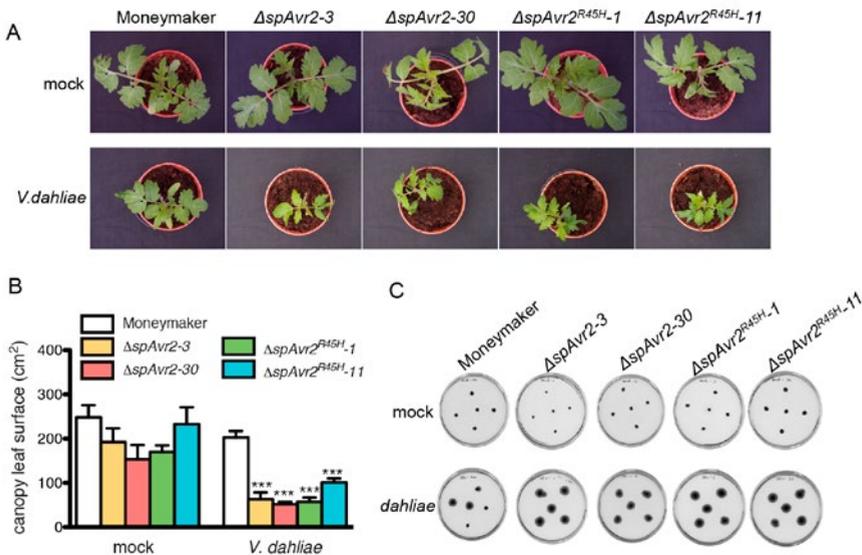
## Results

### 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 ( $\Delta\text{spAvr2}$ ), effectively complements fungal virulence of a *Fo*/ $\Delta\text{Avr2}$  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  $\Delta\text{spAvr2}$  and  $\Delta\text{spAvr2}^{\text{R45H}}$  transgenic tomato plants were inoculated with spores of a race isolate 1 of *V. dahliae* (strain JR2). Besides wild-type Moneymaker, two independent  $\Delta\text{spAvr2}$ -expressing tomato lines ( $\Delta\text{spAvr2-3}$  and  $\Delta\text{spAvr2-30}$ ) and two  $\Delta\text{spAvr2}^{\text{R45H}}$  expressing tomato lines ( $\Delta\text{spAvr2}^{\text{R45H-1}}$  and  $\Delta\text{spAvr2}^{\text{R45H-11}}$ ) 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  $\Delta\text{spAvr2}$  and  $\Delta\text{spAvr2}^{\text{R45H}}$  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

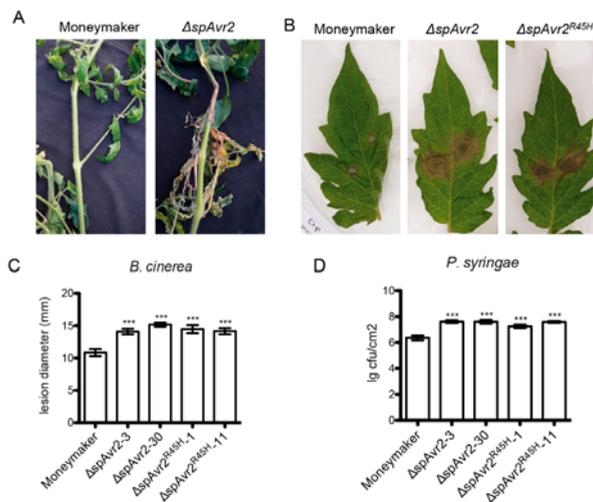


**Figure 1.  $\Delta\text{spAvr2}$  and  $\Delta\text{spAvr2}^{\text{R45H}}$  transgenic tomato plants show enhanced susceptibility to *V. dahliae*.** (A) Representative pictures of mock (upper row) and race 1 JR2 (bottom row) inoculated Moneymaker and  $\Delta\text{spAvr2}$  and  $\Delta\text{spAvr2}^{\text{R45H}}$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

$\Delta spAvr2$  and  $\Delta spAvr2^{R45H}$  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***

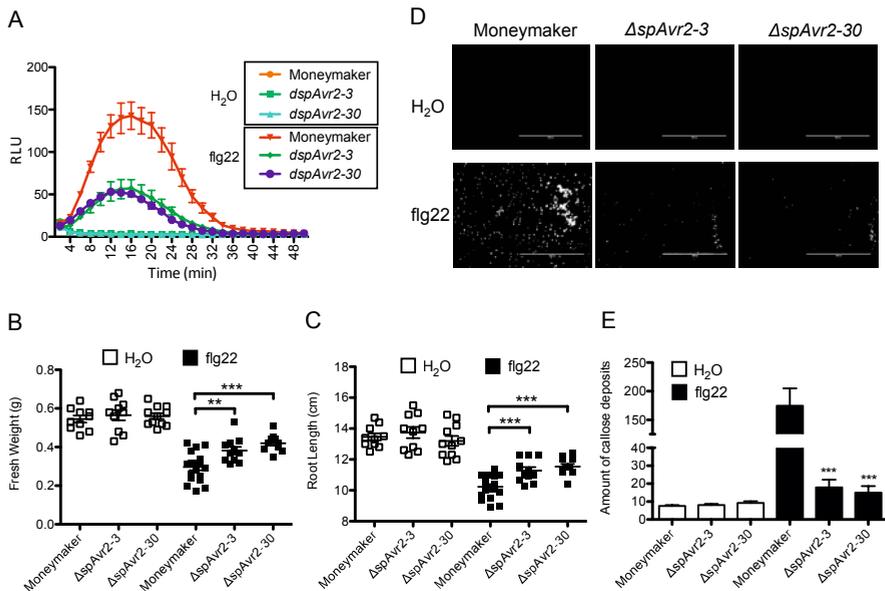
To test whether the virulence-promoting activity of Avr2 extends to pathogens other than the fungal pathogens tested, notably *Fo*, *V. dahliae* and *B. cinerea*, its ability to enhance virulence to a bacterial pathogen was assessed. Thereto susceptibility to infection with *Pseudomonas syringae* pv. *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<sup>2</sup>) in the  $\Delta spAvr2$  and  $\Delta spAvr2^{R45H}$  lines when compared to Moneymaker (Figure 2D). In summary,  $\Delta spAvr2$  and  $\Delta spAvr2^{R45H}$  expression do increase susceptibility of tomato to *Pst*.

### **ROS production and callose deposition are reduced in $\Delta spAvr2$ plants**

Since expression of  $\Delta spAvr2$  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  $\Delta spAvr2$  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 leaf 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  $\pm 150$  RLUs at 16 minutes. Compared to the wild-type Moneymaker both  $\Delta spAvr2$  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  $\Delta spAvr2$  plants, suggesting that PTI signaling is compromised in these plants.

### Avr2 represses seedlings growth inhibition induced by flg22 treatment

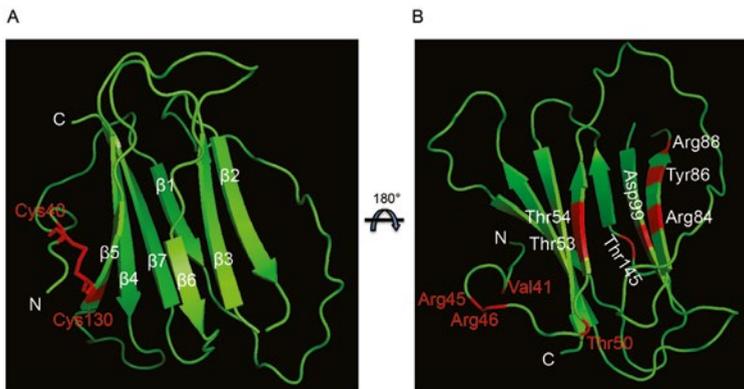
To further determine whether Avr2 possesses PTI-inhibitory activity, seedling-growth-inhibition induced by flg22 treatment was monitored in  $\Delta spAvr2$  transgenic tomato as described (Pfund et al., 2004). Fresh weight and root length were recorded ten days after transferring the plants to liquid 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  $\Delta spAvr2$  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  $\Delta spAvr2$  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  $\Delta spAvr2$  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  $\Delta spAvr2$  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<sub>2</sub>O 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).

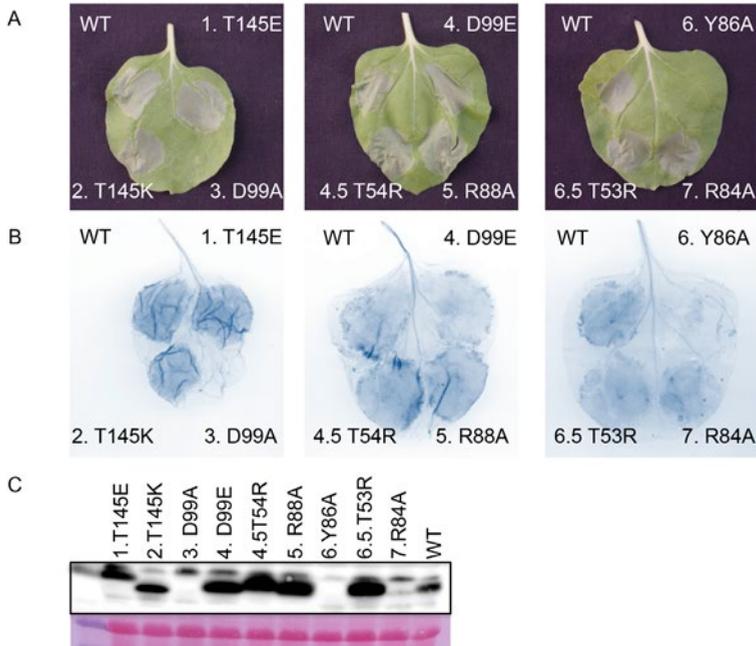
### Site-directed mutagenesis of Avr2 allows uncoupling of its virulence from its avirulence function

Very recently, the crystal structure of Avr2 has been resolved (Di et al., 2017)(Figure 4A). Avr2 forms a  $\beta$ -sandwich fold with two antiparallel  $\beta$ -sheets. The strands are numbered sequentially from  $\beta 1$  to  $\beta 7$  with sheet I composed of strands  $\beta 1$ ,  $\beta 7$ ,  $\beta 4$  and  $\beta 5$  and sheet II containing strands  $\beta 2$ ,  $\beta 3$  and  $\beta 6$ . 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<sup>V41M</sup>, Avr2<sup>R45H</sup>, Avr2<sup>R46P</sup> and Avr2<sup>T50-</sup>) (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  $\beta$ -sandwich fold with two antiparallel  $\beta$ -sheets. The strands are numbered sequentially from  $\beta 1$  to  $\beta 7$  with sheet I composed of strands  $\beta 1$ ,  $\beta 7$ ,  $\beta 4$  and  $\beta 5$  and sheet II containing strands  $\beta 2$ ,  $\beta 3$  and  $\beta 6$ . 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.

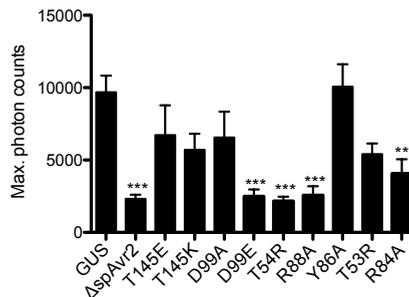
selected that are important for the function of these structurally related proteins and their interaction with either substrates or cofactors. Based on SIAH1 (SINA domain) and its interaction with USP19 (ubiquitin specific peptidase), Avr2 mutants were generated in which the threonine residues at either 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<sup>T53R</sup> and Avr2<sup>T54R</sup>), respectively. Both threonines are located on the same face of the protein as the protruding loop harboring the residues (Avr2<sup>V41M</sup>, Avr2<sup>R45H</sup>, Avr2<sup>R46P</sup> and Avr2<sup>T50</sup>) 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<sup>T145E</sup>, Avr2<sup>T145K</sup>, Avr2<sup>D99E</sup>, Avr2<sup>D99A</sup>, Avr2<sup>R88A</sup>, Avr2<sup>T86A</sup> and Avr2<sup>R84A</sup> were generated (Figure 4B). The mutants are designed to either replace non-charged residues for charged ones (Avr2<sup>T145E</sup>, Avr2<sup>T145K</sup>, Avr2<sup>D99E</sup>, Avr2<sup>T53R</sup> and Avr2<sup>T54R</sup>), or *vice versa* (Avr2<sup>D99A</sup>, Avr2<sup>R88A</sup>, Avr2<sup>T86A</sup> and Avr2<sup>R84A</sup>) 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 of 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<sup>V41M</sup>, Avr2<sup>R45H</sup> and Avr2<sup>R46P</sup>) triggers an HR in this system (Houterman et al., 2009). As shown in Figures 5A and 5B, wild-type  $\Delta$ spAvr2 elicits a clear I-2-dependent cell death response and serves as positive control in these experiments. Two of the nine  $\Delta$ spAvr2 mutants tested (Avr2<sup>D99A</sup> and Avr2<sup>Y86A</sup>) 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<sup>T145E</sup>, Avr2<sup>T145K</sup> and Avr2<sup>R84A</sup>, following *Agrobacterium*-mediated expression (Figure 5C). Notably, for Avr2<sup>D99E</sup>, Avr2<sup>T54R</sup>, Avr2<sup>R88A</sup> and Avr2<sup>T53R</sup> protein levels appeared to be slightly higher than that of the wild-type effector, suggesting the mutations might stabilize the protein. The Avr2<sup>D99A</sup> and Avr2<sup>Y86A</sup> 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. benthamiana* 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<sup>R99A</sup> and Avr2<sup>Y86A</sup> mutants did not suppress ROS formation following flg22 treatment. Mutants Avr2<sup>D99E</sup>, Avr2<sup>T54R</sup> and Avr2<sup>R88A</sup>, 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<sup>T145K/E</sup> and Avr2<sup>T53R</sup>, 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  $\Delta spAvr2$  and  $\Delta spAvr2^{R45H}$  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<sup>V41M</sup>, Avr2<sup>R45H</sup>, Avr2<sup>R46P</sup> and Avr2<sup>T50-</sup>) 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<sup>V41M</sup>, Avr2<sup>R45H</sup>, Avr2<sup>R46P</sup> and Avr2<sup>T50-</sup> 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<sup>T53</sup> and Avr2<sup>T145</sup>) in Avr2 that are essential for virulence, but not for avirulence. The mutations in variants Avr2<sup>D99A</sup> and Avr2<sup>Y86A</sup> 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<sup>T53R</sup> and Avr2<sup>T145</sup> failed to suppress PTI. The Avr2<sup>T53R</sup> and Avr2<sup>T145</sup> 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 (*Fol* $\Delta$ *Avr2*) has been described previously (Houterman et al., 2009).

#### Construction of binary vectors

$\Delta$ *spAvr2*<sup>R45H</sup> was amplified with primers FP2525 and FP2274 using CTAPi:: $\Delta$ *spAvr2*<sup>R45H</sup> 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:: $\Delta$ *spAvr2*<sup>R45H</sup>. SLDB3104:: $\Delta$ *spAvr2* 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

Money maker 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  $\Delta$ *spAvr2* and  $\Delta$ *spAvr2*<sup>R45H</sup> were selected on 1/2 Murashige and Skoog (MS) medium containing kanamycin (40mg/L). To select  $\Delta$ *spAvr2* 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 ( $\Delta$ *spAvr2*-3 and  $\Delta$ *spAvr2*-30). Homozygous  $\Delta$ *spAvr2*<sup>R45H</sup> lines were screened by the same procedure. In the end, two of 17  $\Delta$ *spAvr2*<sup>R45H</sup> plants ( $\Delta$ *spAvr2*<sup>R45H</sup>-1 and  $\Delta$ *spAvr2*<sup>R45H</sup>-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<sup>6</sup> 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,  $5 \times 10^6$  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<sub>4</sub> to an Optical Density (OD)<sub>600</sub> 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<sub>4</sub>. Serial dilutions (typically 10<sup>-3</sup>, 10<sup>-4</sup>, and 10<sup>-5</sup>) 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<sub>2</sub>O. 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<sub>2</sub>HPO<sub>4</sub> 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<sub>2</sub>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<sub>2</sub>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 site directed mutants were generated using quick-change mutagenesis (Zheng et al., 2004). As template for mutagenesis pDONR207::Δ*SpAvr2* 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 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<sub>2</sub>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.

## Acknowledgements

We are gratefully to Mark Banfield and Richard Hughes (John Innes Centre, Norwich, UK) for resolving the structure of Avr2. We thank Bart Thomma and Jan van Kan (WUR, Wageningen, The Netherlands) for providing the *V. dahliae* race 1 JR2 strain and wild-type *B. cinerea* strain B05.10, respectively. The authors wish to thank Ben Cornelissen for critically reading and commenting on the manuscript. X. Di was financially supported by the China Scholarship Council program. FT received funding from the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No. 676480 (Bestpass) and from the NWO-Earth and Life Sciences funded VICI project No. 865.14.003.

**Table 2. Primers used in this study**

Name	Target gene	Sequences (5'-3')
FP2525	$\Delta$ spAvr2 <sup>R45H</sup> -F	CGCTCTAGAATGCCTGTGGAAGATGCCGAT
FP2274	$\Delta$ spAvr2 <sup>R45H</sup> -R	GCGGGATCCTCCATCCTCTGAGATAGTAAG
FP962	Avr2-F	TGAGCGGGCTGGCAATTC
FP963	Avr2-R	CAATCCTCTGAGATAGTAAG
FP6915	Avr2 <sup>T145E</sup> -F	GCTCTCGAGGTCCAGCCGAGATCAGTTGGGATGCCGA
FP6916	Avr2 <sup>T145E</sup> -R	TCGGCATCCCACTGATCTCGGCTGGACCTCGAGAGC
FP6917	Avr2 <sup>T145K</sup> -F	GCATCCCACTGATTTTGGCTGGACCTCGAG
FP6918	Avr2 <sup>T145K</sup> -R	CTCGAGGTCCAGCCAAAATCAGTTGGGATGC
FP6919	Avr2 <sup>D99A</sup> -F	GGGGGGCGACAGCAATGACAGTGCGGAG
FP6920	Avr2 <sup>D99A</sup> -R	CTCCGCACTGTCATTGCTGTGCCCCCC
FP6921	Avr2 <sup>D99E</sup> -F	GGGGGGCGACCTCAATGACAGTGCGGA
FP6922	Avr2 <sup>D99E</sup> -R	TCCGCACTGTCATTGAGGTGCCCCCC
FP6923	Avr2 <sup>T54R</sup> -F	GGTGCTGAAGCTCCTAGTAAATGAAGTAGAAGACGTGC
FP6924	Avr2 <sup>T54R</sup> -R	GCACGTCTTCTACTTCATTTACTAGGAGCTTCAGCACC
FP6925	Avr2 <sup>R88A</sup> -F	CTCCAACGCGACTTGCTTCGTAATGCGGTGATTCAGTCC
FP6926	Avr2 <sup>R88A</sup> -R	GGACTGAATCACCGCATTACGAAGCAAGTCGCGTTGGAG
FP6927	Avr2 <sup>Y86A</sup> -F	GCGACTTCGTTCCGCAATGCGGTGATTGATCCCGCAAT
FP6928	Avr2 <sup>Y86A</sup> -R	ATTCGGGACTGAATCACCGCATTGCCGAACGAAGTCGC
FP6929	Avr2 <sup>T53R</sup> -F	TGCTGAAGCTCGTCCTAAATGAAGTAGAAGACGTGCCGGC
FP6930	Avr2 <sup>T53R</sup> -R	CGCCGCACGTCTTCTACTTCATTTAGGACGAGCTTCAGCA
FP6931	Avr2 <sup>R84A</sup> -F	GCGACTTCGTTGTAATGCGGTGATTGATCCCGCAATTG
FP6932	Avr2 <sup>R84A</sup> -R	CAATTCGGGACTGAATCACGCCATTTACGAACGAAGTCGC

## References

- Bent, A. F. and Mackey, D. (2007) Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. *Annu Rev Phytopathol*, 45, 399-436.
- Boller, T. and Felix, G. (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual review of plant biology*, 60, 379-406.
- Bos, J. I., Armstrong, M. R., Gilroy, E. M., Boevink, P. C., Hein, I., Taylor, R. M., Zhendong, T., Engelhardt, S., Vetukuri, R. R., Harrower, B., Dixelius, C., Bryan, G., Sadanandom, A., Whisson, S. C., Kamoun, S. and Birch, P. R. (2010) *Phytophthora infestans* effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. *Proc Natl Acad Sci USA*, 107, 9909-9914.
- Chellappan, B. V., Fokkens, L., Houterman, P. M., Rep, M. and Cornelissen, B. J. C. (2016) Multiple evolutionary trajectories have led to the emergence of races in *Fusarium oxysporum* f. sp. *lycopersici*. *Appl Environ Microbiol*.
- Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J. D., Felix, G. and Boller, T. (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature*, 448, 497-500.
- Chisholm, S. T., Coaker, G., Day, B. and Staskawicz, B. J. (2006) Host-microbe interactions: shaping the evolution of the plant immune response. *Cell*, 124, 803-814.
- Cortina, C. and Culianez-Macia, F. A. (2004) Tomato transformation and transgenic plant production. *Plant Cell Tiss Org*, 76, 269-275.
- di Pietro, A., Madrid, M. P., Caracuel, Z., Delgado-Jarana, J. and Roncero, M. I. (2003) *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. *Mol Plant Pathol*, 4, 315-325.
- Di, X., Gomila, J., Ma, L., van den Burg, H. A. and Takken, F. L. W. (2016) Uptake of the *Fusarium* effector Avr2 by tomato is not a cell autonomous event. *Front Plant Sci*, 7: 1915. doi: 10.3389/fpls.2016.01915
- 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. (Submitted for publication)
- Dodds, P. N. and Rathjen, J. P. (2010) Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat Rev Genet*, 11, 539-548.
- Fradin, E. F., Zhang, Z., Juarez Ayala, J. C., Castroverde, C. D., Nazar, R. N., Robb, J., Liu, C. M. and Thomma, B. P. (2009) Genetic dissection of *Verticillium* wilt resistance mediated by tomato Ve1. *Plant Physiol*, 150, 320-332.
- Gawehns, F., Houterman, P. M., Ichou, F. A., Michielse, C. B., Hijdra, M., Cornelissen, B. J. C., Rep, M. and Takken, F. L. W. (2014) The *Fusarium oxysporum* effector Six6 contributes to virulence and suppresses I-2-mediated cell death. *Mol Plant Microbe In*, 27, 336-348.
- Gomez-Gomez, L., Felix, G. and Boller, T. (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant Journal*, 18, 277-284.
- Hecht, V., Vielle-Calzada, J. P., Hartog, M. V., Schmidt, E. D., Boutilier, K., Grossniklaus, U. and de Vries, S. C. (2001) The *Arabidopsis* *SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1* gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiol*, 127, 803-816.
- Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J. and Schilperoort, R. A. (1983) A binary plant vector

- strategy based on separation of vir-region and T-region of the *Agrobacterium tumefaciens* Ti-Plasmid. *Nature*, 303, 179-180.
- Houterman, P. M., Cornelissen, B. J. C. and Rep, M. (2008) Suppression of plant resistance gene-based immunity by a fungal effector. *PLoS Pathog*, 4, e1000061.
- Houterman, P. M., Ma, L., van Ooijen, G., de Vroomen, M. J., Cornelissen, B. J. C., Takken, F. L. W. and Rep, M. (2009) The effector protein Avr2 of the xylem-colonizing fungus *Fusarium oxysporum* activates the tomato resistance protein I-2 intracellularly. *Plant J*, 58, 970-978.
- Houterman, P. M., Speijer, D., Dekker, H. L., de Koster, C. G., Cornelissen, B. J. C. and Rep, M. (2007) The mixed xylem sap proteome of *Fusarium oxysporum*-infected tomato plants. *Mol Plant Pathol*, 8, 215-221.
- Jones, J. D. and Dangl, J. L. (2006) The plant immune system. *Nature*, 444, 323-329.
- Lewis, J. D., Lee, A. H. Y., Hassan, J. A., Wan, J., Hurley, B., Jhingree, J. R., Wang, P. W., Lo, T., Youn, J. Y., Guttman, D. S. and Desveaux, D. (2013) The Arabidopsis ZED1 pseudokinase is required for ZAR1-mediated immunity induced by the *Pseudomonas syringae* type III effector HopZ1a. *Proc Natl Acad Sci USA*, 110, 18722-18727.
- Lozano-Duran, R., Bourdais, G., He, S. Y. and Robatzek, S. (2014) The bacterial effector HopM1 suppresses PAMP-triggered oxidative burst and stomatal immunity. *New Phytol*, 202, 259-269.
- Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L. and He, P. (2010) A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proc Natl Acad Sci USA*, 107, 496-501.
- Ma, L., Cornelissen, B. J. C. and Takken, F. L. W. (2013) A nuclear localization for Avr2 from *Fusarium oxysporum* is required to activate the tomato resistance protein I-2. *Front Plant Sci*, 4, 94.
- Ma, L., Houterman, P. M., Gawehns, F., Cao, L., Sillo, F., Richter, H., Clavijo-Ortiz, M. J., Schmidt, S. M., Boeren, S., Vervoort, J., Cornelissen, B. J. C., Rep, M. and Takken, F. L. W. (2015) The AVR2-SIX5 gene pair is required to activate I-2-mediated immunity in tomato. *New Phytol*, 208, 507-518.
- Ma, L., Lukasik, E., Gawehns, F. and Takken, F. L. (2012) The use of agroinfiltration for transient expression of plant resistance and fungal effector proteins in *Nicotiana benthamiana* leaves. *Methods Mol Biol*, 835, 61-74.
- Manning, V. A., Hamilton, S. M., Karplus, P. A. and Ciuffetti, L. M. (2008) The Arg-Gly-Asp-containing, solvent-exposed loop of *Ptr* ToxA is required for internalization. *Mol Plant Microbe In*, 21, 315-325.
- Marino, D., Peeters, N. and Rivas, S. (2012) Ubiquitination during plant immune signaling. *Plant Physiol*, 160, 15-27.
- Melotto, M., Underwood, W., Koczan, J., Nomura, K. and He, S. Y. (2006) Plant stomata function in innate immunity against bacterial invasion. *Cell*, 126, 969-980.
- Michielse, C. B. and Rep, M. (2009) Pathogen profile update: *Fusarium oxysporum*. *Mol Plant Pathol*, 10, 311-324.
- Nicaise, V., Roux, M. and Zipfel, C. (2009) Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. *Plant Physiol*, 150, 1638-1647.
- Park, C. H., Chen, S., Shirsekar, G., Zhou, B., Khang, C. H., Songkumarn, P., Afzal, A. J., Ning, Y., Wang, R., Bellizzi, M., Valent, B. and Wang, G. L. (2012) The *Magnaporthe oryzae* effector AvrPiz-t targets the RING E3 ubiquitin ligase APIP6 to suppress pathogen-associated molecular pattern-triggered immunity in rice. *Plant Cell*, 24, 4748-4762.
- Pfund, C., Tans-Kersten, J., Dunning, F. M., Alonso, J. M., Ecker, J. R., Allen, C. and Bent, A. F. (2004)

- Flagellin is not a major defense elicitor in *Ralstonia solanacearum* cells or extracts applied to *Arabidopsis thaliana*. *Mol Plant Microbe In*, 17, 696-706.
- Rep, M., Meijer, M., Houterman, P. M., van der Does, H. C. and Cornelissen, B. J. C. (2005) *Fusarium oxysporum* evades I-3-mediated resistance without altering the matching avirulence gene. *Mol Plant Microbe In*, 18, 15-23.
- Rep, M., van der Does, H. C., Meijer, M., van Wijk, R., Houterman, P. M., Dekker, H. L., de Koster, C. G. and Cornelissen, B. J. C. (2004) A small, cysteine-rich protein secreted by *Fusarium oxysporum* during colonization of xylem vessels is required for I-3-mediated resistance in tomato. *Mol Microbiol*, 53, 1373-1383.
- Rohila, J. S., Chen, M., Cerny, R. and Fromm, M. E. (2004) Improved tandem affinity purification tag and methods for isolation of protein heterocomplexes from plants. *Plant Journal*, 38, 172-181.
- Schwessinger, B. and Ronald, P. C. (2012) Plant innate immunity: perception of conserved microbial signatures. *Annual review of plant biology*, 63, 451-482.
- Shi, H., Shen, Q., Qi, Y., Yan, H., Nie, H., Chen, Y., Zhao, T., Katagiri, F. and Tang, D. (2013) BR-SIGNALING KINASE1 physically associates with FLAGELLIN SENSING2 and regulates plant innate immunity in *Arabidopsis*. *Plant Cell*, 25, 1143-1157.
- Simons, G., Groenendijk, J., Wijbrandi, J., Reijans, M., Groenen, J., Diergaarde, P., Van der Lee, T., Bleeker, M., Onstenk, J., de Both, M., Haring, M., Mes, J., Cornelissen, B., Zabeau, M. and Vos, P. (1998) Dissection of the *Fusarium* *l2* gene cluster in tomato reveals six homologs and one active gene copy. *Plant Cell*, 10, 1055-1068.
- Takken, F. L. W. and Rep, M. (2010) The arms race between tomato and *Fusarium oxysporum*. *Mol Plant Pathol*, 11, 309-314.
- Tameling, W. I. L., Nooijen, C., Ludwig, N., Boter, M., Slootweg, E., Goverse, A., Shirasu, K. and Joosten, M. H. A. J. (2010) RanGAP2 mediates nucleocytoplasmic partitioning of the NB-LRR immune receptor Rx in the Solanaceae, thereby dictating Rx function. *Plant Cell*, 22, 4176-4194.
- Thomma, B. P., Nurnberger, T. and Joosten, M. H. (2011) Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *Plant Cell*, 23, 4-15.
- van Ooijen, G., Mayr, G., Kasiem, M. M., Albrecht, M., Cornelissen, B. J. and Takken, F. L. (2008) Structure-function analysis of the NB-ARC domain of plant disease resistance proteins. *J Exp Bot*, 59, 1383-1397.
- van Ooijen, G., van den Burg, H. A., Cornelissen, B. J. C. and Takken, F. L. W. (2007) Structure and function of resistance proteins in solanaceous plants. *Annu Rev Phytopathol*, 45, 43-72.
- Veronese, P., Nakagami, H., Bluhm, B., Abuqamar, S., Chen, X., Salmeron, J., Dietrich, R. A., Hirt, H. and Mengiste, T. (2006) The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens. *Plant Cell*, 18, 257-273.
- Weissman, A. M. (2001) Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol*, 2, 169-178.
- Whalen, M. C., Innes, R. W., Bent, A. F. and Staskawicz, B. J. (1991) Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell*, 3, 49-59.
- Xie, P. (2013) TRAF molecules in cell signaling and in human diseases. *J Mol Signal*, 8, 7.
- Zhang, J., Li, W., Xiang, T., Liu, Z., Laluk, K., Ding, X., Zou, Y., Gao, M., Zhang, X., Chen, S., Mengiste, T., Zhang, Y. and Zhou, J. M. (2010) Receptor-like cytoplasmic kinases integrate signaling from multiple plant immune receptors and are targeted by a *Pseudomonas syringae* effector. *Cell Host Microbe*, 7, 290-301.

- Zhang, L. S. and Van Kan, J. A. L. (2013) *Botrytis cinerea* mutants deficient in D-galacturonic acid catabolism have a perturbed virulence on *Nicotiana benthamiana* and Arabidopsis, but not on tomato. *Mol Plant Pathol*, 14, 19-29.
- Zheng, L., Baumann, U. and Reymond, J. L. (2004) An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res*, 32, e115.
- Zhuang, M., Calabrese, M. F., Liu, J., Waddell, M. B., Nourse, A., Hammel, M., Miller, D. J., Walden, H., Duda, D. M., Seyedin, S. N., Hoggard, T., Harper, J. W., White, K. P. and Schulman, B. A. (2009) Structures of SPOP-substrate complexes: insights into molecular architectures of BTB-Cul3 ubiquitin ligases. *Mol Cell*, 36, 39-50.