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

Ma, L.

Publication date
2012

CHAPTER 5

A pair of avirulence factors is required to activate the immune system of tomato

Abstract

Plant-invading microbes betray their presence to a plant by exposure of secreted molecules such as effector proteins. We have identified an effector pair, notably Six5-Avr2, in the fungus *Fusarium oxysporum* that is recognized by a single resistance gene in tomato, *I-2*. Expression of this effector gene pair is controlled by a shared promoter. Knockout of either gene compromises *I-2* mediated resistance and partially impairs virulence. Whereas the effector pair is required for *I-2* mediated resistance, Avr2 alone is sufficient to trigger *I-2*-mediated cell death suggesting that cell death and resistance can be uncoupled. Both effectors interact and *in planta* accumulation of Six5 might require the presence of Avr2. To the best of our knowledge this is the first report of a pair of effectors being recognized by a single R gene.

A manuscript is prepared as:

L. Ma*, P.M. Houterman*, F. Sillo, H. Richter, S. Boeren, M.J. Clavijo Ortiz, S. Schmidt, F. Gawehns, M. de Sain, H. Dekker, B.J.C. Cornelissen, M. Rep, F.L.W. Takken

* Equal contribution

Introduction

Vascular wilt diseases caused by fungi are among the most devastating plant diseases worldwide. There are only a limited number of fungal species that can cause vascular wilt diseases and most of them belong to the genera *Fusarium*, *Verticillium* or *Ophiostoma* (Tjamos, 1989). After infecting the vasculature these pathogens are shielded from most fungicides, prohibiting curative treatments of infected plants. Because of persistent resting structures, such as chlamydospores, and the ability to grow saprophytically, these fungi exhibit extreme longevity in the soil (decades) making crop-rotation futile (Michielse and Rep, 2009). The use of resistant plant varieties is used as a strategy to control vascular wilt diseases in some crops. Unfortunately, for many crops no natural resistances are known and when resistances are available, they can be overcome by the pathogen in the course of time (Michielse and Rep, 2009). The high economic impact of wilt diseases combined with the lack of sustainable control measures substantiate the need for a better understanding of the molecular mechanisms underlying disease and resistance to vascular pathogens. The interaction between tomato and *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) has emerged as a model system to study these mechanisms (Takken and Rep, 2010).

To defend themselves against pathogens plants rely on a sophisticated innate immune system. Two distinct layers can be distinguished: the first is activated by perception of conserved pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors. This basal immune response (also called PAMP Triggered Immunity - PTI) wards off attacks by most microbes. Many pathogenic microbes subvert PTI via "effector" proteins that manipulate specific host-targets (Dodds and Rathjen, 2010). Some plants however, carry "resistance" proteins that recognise specific effectors, followed by the initiation of the second layer of defence called effector-triggered immunity (ETI). Pathogens that overcome ETI have often shed or mutated an effector to prevent recognition by the corresponding resistance gene. Based on the correlation between an effector gene in the pathogen and a corresponding resistance gene in the host such an interaction is also called gene-for-gene relation. Wilt resistance in tomato against *Fol* complies with the gene-for-gene model. Three distinct *R*-genes, notably *I* (or *I-1*), *I-2* and *I-3*, confer resistance against *Fol* races containing *AVR1*, *AVR2* and *AVR3*, respectively. So far, only *I-2* has been cloned and characterized. Like most *R* genes it encodes a classical NB-LRR protein carrying a central Nucleotide Binding (NB) domain and a Leucine Rich Repeat (LRR) domain. The I-2 protein resides intracellularly, but its precise subcellular localization is as yet unknown.

During host colonisation *Fol* secretes small, cysteine-rich proteins into the xylem sap that bear little or no resemblance to proteins in closely related species. Using mass-spectrometry, our lab identified thirteen of these "Secreted In Xylem" (Six) proteins (Six1-11) in sap isolated from infected tomato plants ((Houterman *et al*., 2007), and unpublished data). Three of these, Six4, Six3 and Avr3, trigger *Fol* resistance mediated by the three

respective tomato *R* genes. Hence these Six proteins have been renamed into Avr1, Avr2 and Avr3, respectively. Although Avr2 was identified in the xylem sap, it activates *I-2* intracellularly, which can be visualised as a cell death response in *Nicotiana benthamiana* as well as in tomato (Houterman *et al*., 2009). Avr2 and Avr3 do not only act as avirulence factors, but are required for full virulence on susceptible plants as well. *AVR1* is not required for virulence on tomato plants without resistance genes against *Fol*. However, *AVR1* knockouts lose pathogenicity on plants containing either *I-2* or *I-3*, suggesting that Avr1 functions as suppressor of *I-2* and *I-3*-mediated resistance (Houterman *et al*., 2008). Recently an *AVR1* (*SIX4*) homolog in the Arabidopsis-infecting strain Fo5176 was found to be required for full virulence on its host Arabidopsis (Thatcher *et al*., 2012). Except for the three aforementioned Six proteins, no clear functions have been assigned to the others. Interestingly, all known *SIX* genes, except for *SIX4* (*AVR1*), reside on a single small and repetitive element-rich chromosome. This chromosome (number 14 in Fol4287) can be transferred from a pathogenic to a non-pathogenic strain during co-cultivation, resulting in the acquisition of pathogenicity by the recipient strain (Ma *et al*., 2010; Rep and Kistler, 2010).

As part of our ongoing functional analysis of Six proteins, we here focus on Six5. Six5 was originally identified as "unidentified protein 2" in the xylem sap proteome of *Fol* infected tomato plants. On a 2D gel the protein runs as a single spot with an apparent mass of 12 KDa (Houterman *et al*., 2007). After identifying the coding sequence in the *Fol* genome sequence (the gene was not annotated) the protein was renamed Six5. The mRNA sequence of 360b is filed in the NCBI dbase under accession number FJ767863 (Ma *et al*., 2010). *SIX5*, together with three other *SIX* genes (*AVR3* (*SIX1*), *AVR2* (*SIX3*) and *SIX2*) are highly conserved in all known *Fol* isolates (Lievens *et al*., 2009). We here show that the protein contributes to virulence of *Fol* on tomato plants and, very surprisingly, Six5 is also required for *I-2*-mediated resistance.

Results

SIX5 and *AVR2* share a bi-directional promoter region

Like the majority of the *Fol SIX* genes, *SIX5* resides on chromosome 14 in the sequenced isolate Fol4287 (Ma *et al.*, 2010; Rep and Kistler, 2010). The *SIX5* gene was found to contain three introns and the gene is located in close proximity of *AVR2 (SIX3)*. Both genes appear to share a promoter region of 1.179 bp (Figure 1). In this region one *mimp* (miniature impala) transposable element is present that is located 230 bp upstream of the *AVR2* ORF. A single promoter that drives transcription into two opposite directions is a feature more frequently observed in eukaryotes in which co-expression of genes often indicates a functional relationship between the gene products (Kensche *et al.*, 2008; Yang *et al.*, 2008).

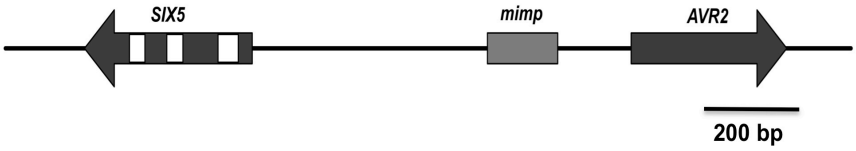

Figure 1. **Schematic representation of the genomic location of *SIX5* and *AVR2*.** *AVR2* and *SIX5* share the same promoter region and are transcribed in opposite directions. White blocks in *SIX5* represent introns; mimp: miniature impala (transposon).

Six5 is required for full virulence of *Fol*

To assess the role of Six5 for the infection process of tomato, *Fol SIX5* knockouts (Δ*SIX5*) were generated by homologous recombination. A hygromycin resistance gene cassette, flanked by 809 and 979 bp of sequences upstream and downstream of the *SIX5* ORF, respectively, was used to replace the *SIX5* gene. Out of 120 hygromycin resistant transformants one genuine *SIX5* deletion mutant was identified by PCR based on the insertion of the hygromycin cassette in the *SIX5* locus and absence of the *SIX5* gene (data not shown).

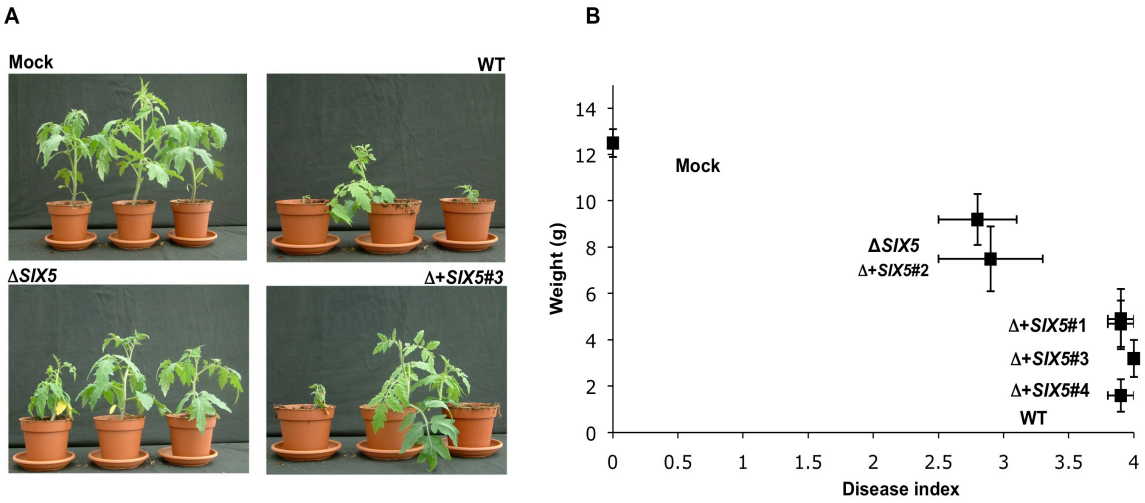

Figure 2. ***SIX5* is required for full virulence of *Fol*.** Ten-day-old seedlings of susceptible tomato line C32 were inoculated with race 2 isolate Fol007 (WT), a mutant derived from Fol007 in which the *SIX5* gene had been deleted (Δ*SIX5*) and the knockout mutant in which *SIX5* was re-introduced (Δ+*SIX5#1-4*). As control, C32 was water-inoculated (Mock) as well. **A)** Representative plants were photographed 3 weeks after inoculation. **B)** Average disease index of 20 plants 3 weeks after inoculation was plotted against the mean weight of those tomato plants. Deletion of *SIX5* impairs the fungal pathogenicity, as shown by the reduced disease index and weight of infected tomato plants when compared to infection with wild-type (WT) control. However, three out of four *SIX5* independent complements displayed the same level disease index and plant weight with WT control. Error bars indicate standard error.

Pathogenicity of the Δ*SIX5* strain was assessed by inoculating roots of ten-day-old C32 tomato seedlings. C32 does not carry resistance genes against *Fol* and inoculation with the wild-type (WT) (Fol007) strain resulted in typical Fusarium disease symptoms such as wilting and stunting (Figure 2A). Disease severity was scored on a scale from 0-4 and plant weight was measured (Figure 2B). The Δ*SIX5* strain displayed reduced virulence as shown by the increased vigour of the plants, their higher weights and lower disease index as compared to the Fol007 (WT) control (Figure 2A and 2B). Re-introduction of *SIX5* into the Δ*SIX5* background (Δ+*SIX5*) restored full pathogenicity in three out of four independent transformants, which confirms that the reduced pathogenicity of Δ*SIX5* is caused by deletion of *SIX5* (Figure 2A and 2B). A fifth transformant completely lost its pathogenicity and was excluded from further analyses. These results show that Six5 is required for full pathogenicity of *Fol* on tomato, identifying Six5 as a genuine effector protein.

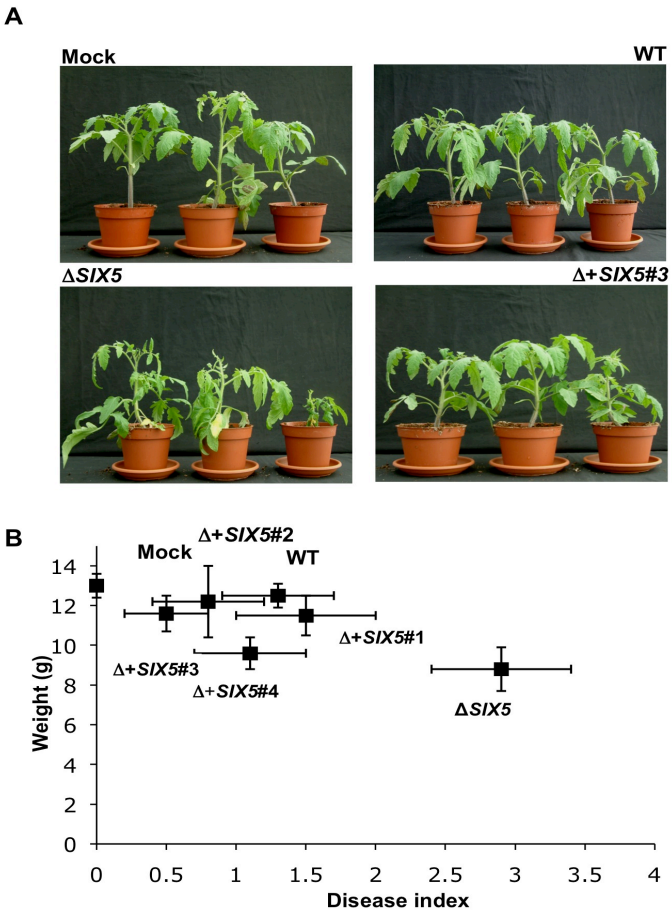

Figure 3. Deletion of *SIX5* evades I-2-mediated resistance. Ten-day-old seedlings of tomato plant line with *I-2* gene were inoculated with race 2 strain Fol007 (WT), a mutant derived from Fol007 in which the *SIX5* gene had been deleted (Δ*SIX5*) and the knockout mutant in which *SIX5* was re-introduced (Δ+*SIX5#1-4*). As a control water-inoculated plant was used (Mock). **A)** Representative plants were photographed 3 weeks after inoculation. **B)** Average disease index of 20 plants was scored and the mean plant weight was measured as well three weeks after mock inoculation. Tomato plants infected with the Δ*SIX5* strain displayed increased disease index and reduced plant weight. Three out of four independent complementants displayed the same level disease index and plant weight with WT control. Error bars indicate standard error.

Six5 and Avr2 both are required for *I-2*-mediated resistance

Avr2 is required for *I-2*-mediated resistance (Houterman *et al.*, 2009), and we wished to investigate whether Six5 is also involved in this recognition process. Tomato seedlings that carry the *I-2* resistance gene were inoculated with the Δ*SIX5* strain. In contrast to Fol007, the Δ*SIX5* strain had acquired the ability to cause disease on *I-2* plants (Figure 3). Re-introduction of *SIX5* into the Δ*SIX5* strain restored avirulence on *I-2* plants in three out of the four tested transformants. These three complementants were the same strains in which virulence was fully restored on the susceptible plants (see above). This result confirms that loss of avirulence is caused by deletion of *SIX5* (Figure 3A and 3B). Apparently, deletion of *SIX5* allowed the fungus to circumvent *I-2* mediated resistance. Deletion of *SIX5* in race 1 isolate Fol004 did not result in a strain that overcomes *I* mediated resistance (data not shown). We conclude that besides *AVR2* also *SIX5* is required for *I-2*-mediated resistance in tomato, revealing a functional interaction between these two genes.

AVR2* expression is independent of *SIX5* expression and *vice versa

One explanation of the requirement of *SIX5* for *I-2*-mediated resistance is that it is required for expression of *AVR2*. To test this possibility, expression of both effector genes was analysed in the reciprocal knockouts. RNA was extracted from roots and stems inoculated

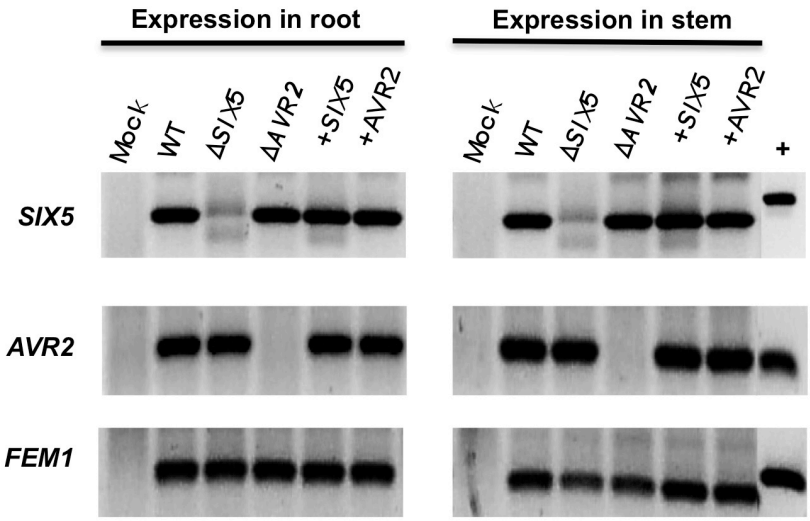

Figure 4. *In planta* expression of *AVR2* and *SIX5*. Ten-day-old tomato seedlings were inoculated with wild-type (WT) *Fol*, the Δ*SIX5* and Δ*AVR2* strains, and complementants, respectively. As a control, water-inoculated tomato was used (Mock). Ten days post inoculation RNA was isolated from roots and stems. RT-PCR was performed on *AVR2*, *SIX5* and *FEM1* (internal control for the amount of *Fol* cDNA). The left panel shows the expression of *AVR2*, *SIX5* and *FEM1* in the infected roots and the right panel shows the expression in stems.

with the knockout strains Δ*AVR2* and Δ*SIX5,* the complemented strains and Fol007. The presence of *SIX5* or *AVR2* transcripts was determined by reverse transcription-polymerase chain reaction (RT-PCR). As shown in Figure 4, *SIX5* and *AVR2* transcripts are readily detected in the WT control in root and stem. Expression of *SIX5* in the Δ*AVR2* was comparable to that of the wild-type, indicating that deletion of *AVR2* did not affect *SIX5* expression. Likewise, deletion of *SIX5* did not affect expression of *AVR2* (Figure 4). We conclude that the *I-2* breaking phenotype of Δ*SIX5* cannot be attributed to a lack of *AVR2* expression.

Accumulation of Six5 in the xylem sap of the Δ*AVR2* strain -inoculated tomato is reduced

The observed functional interaction between Avr2 and Six5 raised the possibility that both proteins form a heteromeric complex in the xylem sap of *Fol*-infected tomato plants. Formation of such a complex might be required for their stabilisation and subsequent accumulation in xylem sap. To test this hypothesis, we inoculated 5-week-old C32 plants with either Fol007, the Δ*AVR2* strain*,* the Δ*SIX5* strain or water, and isolated xylem sap two weeks later. At this stage the first disease symptoms become apparent in the Fol007-inoculated plants. To detect the presence of Avr2 and Six5 in the xylem sap, polyclonal antibodies were raised in rabbits against GST-tagged Avr2 and Six5 proteins that were heterologously produced in *E. coli.* Additionally, an Avr3 antibody was raised to allow detection of an unrelated Six protein whose expression is strongly induced during plant infection (van der Does *et al.*, 2008). The specificity and sensitivity of the antibodies was determined by assessing their ability to detect either HA- or GFP-tagged Six proteins in extracts of agroinfiltrated *N. benthamiana* leaves transiently expressing the corresponding gene (Figure 5). Surprisingly, in the *N. benthamiana* leaf extracts only HA-Avr3 and GFP-Avr2 could be detected. Reprobing the blots using either a GFP or an HA antibody also revealed only the presence of the latter two proteins, indicating that *SIX5* is either not expressed in this system or that accumulation of the protein is below the detection level of both Six5 antibodies (Figure 5).

The generated antibodies were then used to detect the presence of the three Six proteins in the xylem sap of the *Fol*-infected tomato plants described above. As shown in Figure 6, application of the Avr3 antibody resulted in a strong signal for the xylem sap of Fol007-inoculated plants and a much weaker signal for both the Δ*AVR2* and Δ*SIX5* strains at the expected height for Avr3. This lower signal in the knockout strains likely reflects a reduced amount of fungal biomass in the plant, in line with the reduced virulence of the respective knockout strains (Figure 2 and (Houterman *et al.*, 2009)).

The immunoblot probed with the Avr2 antibody revealed accumulation of the Avr2 protein in xylem sap of plants inoculated with Fol007 and the Δ*SIX5* strain*,* but - as expected

- not in sap of plants inoculated with the Δ*AVR2* strain or in the water control. The signal intensity of the Avr2 bands is similar to that of Avr3 suggesting a similar abundance of both effectors. An identical blot was probed with the Six5 antibody and a band at the expected molecular mass of Six5 was clearly detected in xylem sap of Fol007-inoculated plants. As no signal was detected in xylem sap of the Δ*SIX5* strain-inoculated plants, this demonstrates that the Six5 antibody specifically recognises this effector. Notably, also no band was observed in xylem sap of the Δ*AVR2*-infected plants, implying that accumulation of Six5 is reduced.

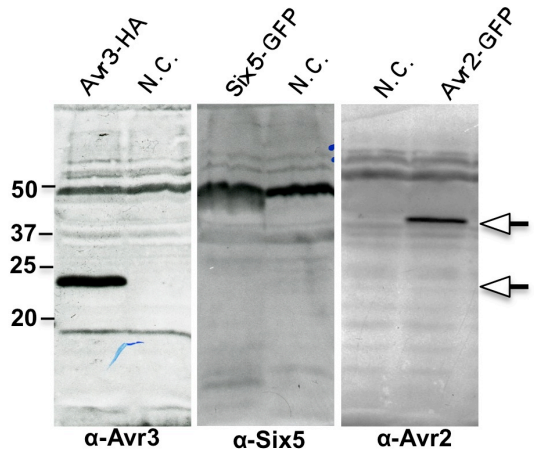

Figure 5. Verification of Avr3, Avr2 and Six5 polyclonal antibodies using protein extracts of *Nicotiana benthamiana* leaves expressing the corresponding effector gene constructs. Proteins isolated from *N. benthamiana* leaves agro-infiltrated to express *AVR2-GFP*, *SIX5-GFP* and *AVR3-HASBP* were subjected to SDS-PAGE, followed by immunoblotting and detection with the corresponding antibody. In both the Avr3-HA and Avr2-GFP lanes a specific signal is detected corresponding to the expected molecular size of the effector proteins. No specific signal was observed in Six5-GFP. N.C.=negative control. Arrows indicate the interesting bands.

To analyse whether Six5 is completely absent or accumulates at levels below the detection level of the antibody, xylem sap proteomes were collected from non-inoculated, Fol007-inoculated and the Δ*AVR2* or Δ*SIX5* strain-infected plants. To precisely quantify the accumulation of effector proteins in xylem sap label-free quantitative proteomics was used. This method allows relative quantification of Six5 and Avr2 by comparing their abundance to that of all other fungal and plant proteins detected in the xylem sap. Per sample approximately 45 µg of protein was recovered from concentrated xylem sap and subjected to SDS-PAGE. After Coomassie blue staining a single band, containing all proteins, was dissected and used for in-gel digestion and subsequent nano liquid chromatography MS/MS (nLC-MS/MS) proteomic measurements and MaxQuant analysis. Four independent biological replicates were performed and the obtained spectra were matched to both the SGN tomato protein database and the Fusarium protein database. The screen retrieved 577 peptides with a hit in either

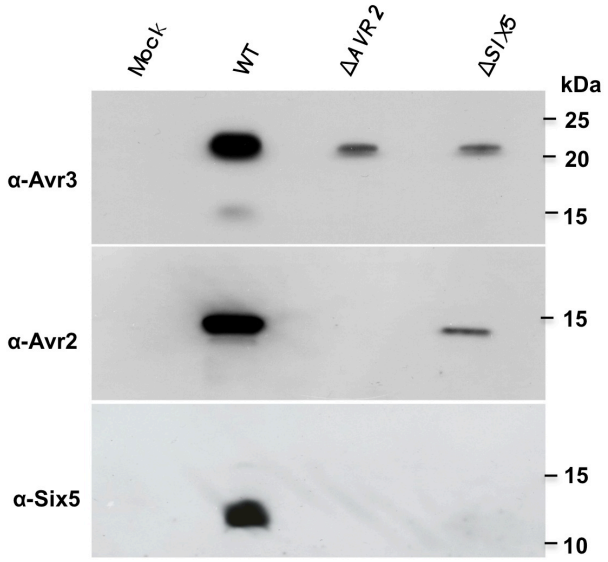

Figure 6. Accumulation of Avr2 and Six5 in xylem sap of infected tomato plants. Four-week-old tomato plants were inoculated with water (Mock), WT and the Δ*SIX5* and Δ*AVR2* strains. Two weeks post inoculation xylem sap was isolated from inoculated plants. Western blot of xylem sap was probed with Avr3, Avr2 and Six5 antibody, respectively. Avr3 protein was taken as standard control of *Fol* proteins and loading.

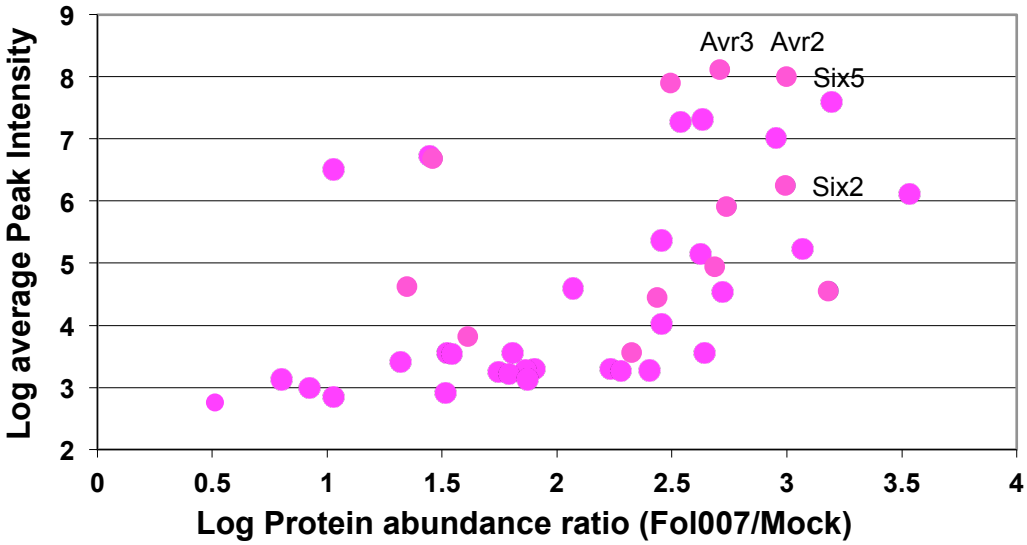

Figure 7. Quantitative analysis of *Fol* proteins present in xylem sap collected from mock-inoculated and Fol007-inoculated tomato. Label free quantification of the xylem sap proteome of four-week-old tomato plants inoculated with water and Fol007 (WT). Four independent replicas were included. Normalized protein abundance ratio (Fol007/Mock) is plotted against the average peptide intensity. Among 42 *Fol* proteins, Avr3, Avr2 and Six5 form a cluster representing the most abundant *Fol* proteins present in the xylem sap and indicating all of them accumulates at the similar level in the xylem sap.

database; 67 of these peptides corresponded to a *Fol* protein, the others either match tomato proteins or contaminants such as the trypsin used for digestion. After filtering for proteins that match with at least two peptides, of which at least one unique one, 455 proteins remained, 42 of which were derived from *Fol* (Figure 7). Avr3, Six5 and Avr2 are among the most abundant fungal proteins present in the sap of WT-infected plants and they cluster

together implying that all three accumulate at the similar level in the xylem sap during *Fol* infection (Figure 7). Next, the relative amount of Avr3, Avr2 and Six5 in the sap of Fol007 (WT), Δ*AVR2* and Δ*SIX5*-infected plants are quantified. Figure 8 shows that the relative amounts of Avr3 and Avr2 are similar in the sap of the Δ*SIX5* strain-inoculated plants. However, the relative amount of Six5 is decreased when compared to that of Avr3 in the sap of the Δ*AVR2* strain-inoculated plants. These data combined with the immunoblotting results imply that Six5 accumulation in xylem sap of Δ*AVR2*-infected plants is reduced suggesting that Avr2 might contribute to accumulation of Six5, but it is not essential.

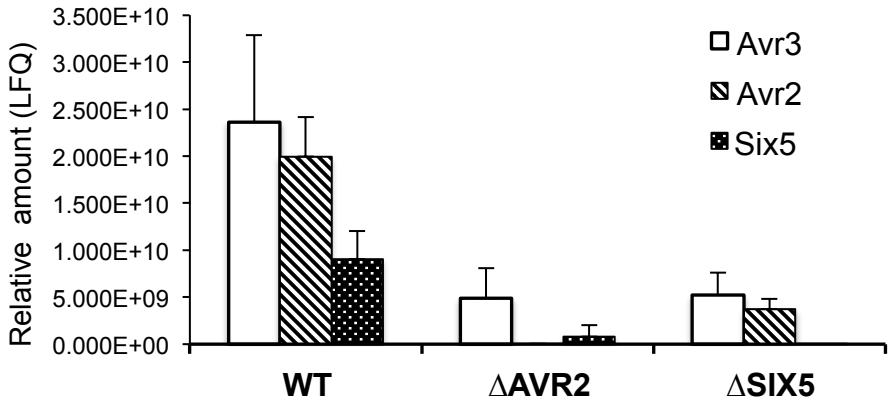

Figure 8. Relative amount of Avr3, Avr2 and Six5 present in the xylem sap of strains Fol007, Δ*AVR2* and Δ*SIX5*-inoculated tomato. Label free quantification of the xylem sap proteome of four-week-old tomato plants inoculated with strains Fol007 (WT), Δ*AVR2* and Δ*SIX5*, respectively. Normalized relative amount (LFQ: label free quantification) of Avr3, Avr2 and Six5 in four independent treatments is shown. Avr3, Avr2 and Six5 all are present in WT-inoculated plant. Avr2 or Six5 is absent in the respective knockouts, but Avr3 is present in both knockouts at similar level. The relative amount of Avr3 in both knockouts was reduced as compared to that in WT resulting from the less fungal biomass in knockout-inoculated plant. The relative abundance of Six5 in the sap of strain Δ*AVR2* inoculated plants was slightly reduced, but the relative amount of Avr2 in the sap of strain Δ*SIX5* inoculated plants is unaffected as compared to the accumulation of Avr3. Error bars indicate the standard error.

Six5 accumulates in xylem sap of *Fol* race 3-inoculated plants

Fol race 3 isolates break I-2-mediated resistance since they carry an *AVR2* variant with a point mutation preventing I-2 activation. Currently, 3 independent I-2 breaking Avr2 isolates have been identified: V41M, R45H and R46P (Houterman *et al.*, 2009). Whereas deletion of *AVR2* reduces virulence, race 3 isolates are still fully pathogenic. Furthermore, the mutant Avr2 variants can restore virulence of an *AVR2* knockout strain, showing that their virulence function is unaffected (Houterman *et al.*, 2009). Since we found that accumulation of Six5 is reduced in absence of Avr2, and Six5 is required for I-2 mediated resistance, we asked whether Six5 accumulation in xylem sap is affected in race 3 isolates. Xylem sap was isolated from non-inoculated, Fol004 (a race 1 isolate), Fol007 (race 2) or Fol029 (a race 3

isolate carrying the *AVR2*^{R45H} variant) inoculated tomato. Immunoblotting with the Avr2 antibody revealed that in xylem sap of plants infected with all three races Avr2 accumulates to similar levels. This observation confirms our earlier observation that the *AVR2*^{R45H} variant is stable and its ability to escape I-2 mediated recognition is not caused due to reduced amounts of the protein in the xylem sap (Figure 9). Interestingly, Six5 was also found to accumulate in comparable amounts in the three *Fol* races (Figure 9). These findings show that breaking of *I-2* mediated resistance by race 3 isolates is not due to the absence of Six5. It is also apparent that Six5 is required, but not sufficient, for I-2 mediated resistance and that induction of resistance requires both effector proteins.

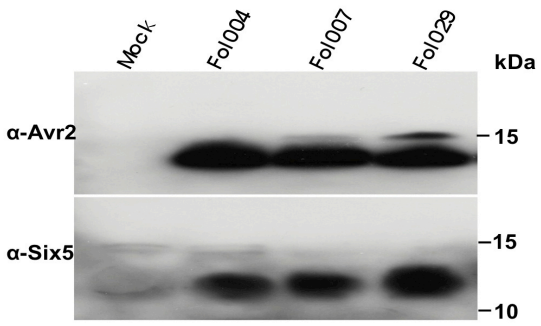

Figure 9. **Accumulation of Avr2 and Six5 in xylem sap of tomato inoculated with different *Fol* races.** Xylem sap was isolated from tomato inoculated with Fol004 (race 1), Fol007 (race 2) and the *I-2* braeking isolate Fol029 (race 3). Xylem sap was subjected to Tricine-SDS, followed by immunoblotting and probing with Avr2 or Six5 antibody. The mock-labeled lane represents xylem sap isolated from water-inoculated tomato plants. In all races Avr2 and Six5 accumulate to similar levels showing that the ability to break *I-2* is not correlated with a lack of Six5 accumulation in the Fol029 strain.

Six5 and Avr2 physically interact with each other

Since Six5 and Avr2 are both required for *I-2* mediated resistance and Six5 requires Avr2 for accumulation, we set out to examine whether there is a physical interaction between these two proteins. For this we exploited the GAL4-based yeast two-hybrid system in which *AVR2* or *AVR2*^{R45H} was expressed as bait and Six5 as prey protein. Transformed yeast cells containing the bait and prey plasmids were selected on plates lacking Trypthophan and Leucine. Interaction between the two effectors would allow the yeast to grow on medium lacking histidine. As shown in Figure 10A, Six5 nor Avr2 or Avr2^{R45H} alone are able to complement the histidine auxotrophy in yeast, while in combination they enable growth on plates lacking histidine, but no growth on the more stringent selection plates lacking adenine. This suggests that Avr2 or its variant Avr2^{R45H} can physically interact with Six5. Expression of bait and prey proteins was confirmed by immunoblot probed with either the Avr2 antibody or a HA antibody recognising HA-tagged Six5 (Figure 10B).

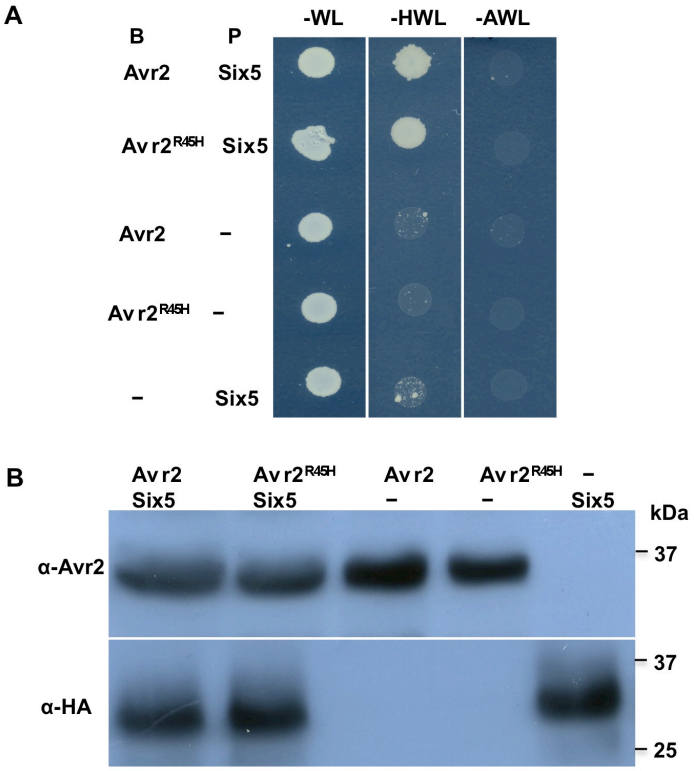

Figure 10. Six5 physically interacts with Avr2 in the yeast two-hybrid system. A) Avr2 interacts with Six5 weakly in the GAL4 yeast two-hybrid system. Transformed yeast pPJ694a strains with the *AVR2* or *AVR2*^{R45H} construct in pAS2-1 or empty vector as bait (b) and *SIX5* construct in pACT2 or empty vector as prey (p) were grown on the –WL plates lacking Trp and Leu, -HWL plates lacking Trp, Leu and His, and –AWL plates lacking Ade, Trp and Leu (-WL selections for the presence of bait and prey plasmids, -HWL selection for weak interaction, -AWL selection for the strong interaction). **B)** Expression of baits and prey proteins in the yeast two-hybrid system. Proteins were extracted from yeast grown in –WL medium and subjected to SDS-PAGE, followed by immunoblot and detection with Avr2 or HA antibody. Presence of bait Avr2 or its Avr2^{R45H} variant was detected in the corresponding yeast transformants (upper panel). Similar, Six5-HA was detected in the strains expressing SIX5 (lower panel).

In a heterologous system Six5 cannot activate *I-2* to trigger cell death

Activation of R proteins by effectors induces host defences, which are often accompanied by a local cell death response (Dodds and Rathjen, 2010; Jones and Dangl, 2006). Although a cell death response is typically not observed upon invasion of roots or xylem by an avirulent *Fol* isolate (Houterman *et al.*, 2009; Mes *et al.*, 2000), a cell death response can be triggered upon heterologous co-expression of *AVR2* and *I-2* in *Nicotiana benthamiana* leaves (Houterman *et al.*, 2009; Mes *et al.*, 2000). To examine whether Six5 can trigger an *I-2*-dependent cell death response in this system, *SIX5* and *I-2* were co-expressed in *N. benthamiana* leaves using agroinfiltration. Since I-2 is an intracellular protein and Avr2 is recognised inside the plant cell, the sequence coding for the signal peptide for secretion in *SIX5* was deleted (Δsp). As shown in Figure 11A, co-expression of Δsp*SIX5* and *I-2* did not trigger cell death, showing that Six5 is unable to trigger *I-2*-mediated cell death.

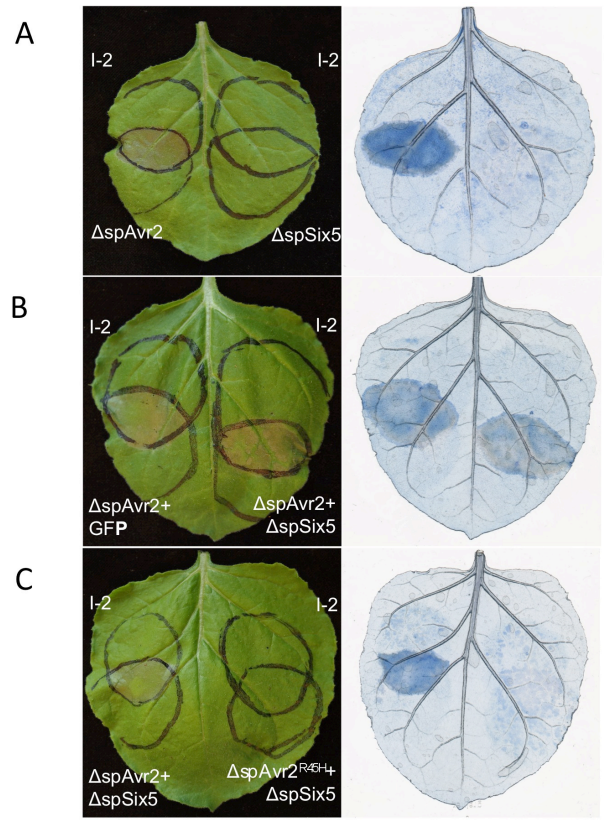

Figure 11. Avr2, but not Six5, triggers *I-2*-mediated cell death in *Nicotiana benthamiana* leaves when co-expressed via agroinfiltration. A) Co-expression of *I-2* and *AVR2* lacking its signal peptide for secretion (ΔspAvr2), triggers cell death (necrotic sector) that can be visualized using a trypan blue staining. Co-expression of *I-2* and *ΔspSIX5* does not induce cell death. **B)** Co-expression of *ΔspSIX5* with *ΔspAVR2* and *I-2* does not lead to enhanced cell death as compared to the GFP control. **C)** ΔspSix5 does not induce cell death when co-expressed with the *ΔspAvr2*^{R45H} variants and I-2 demonstrating that Six5 cannot trigger I-2-mediated cell death.

Co-expression of Δsp*AVR2* and *I-2* did induce a robust cell death response (Figure 11A), confirming our earlier observations that Avr2 alone is sufficient to trigger *I-2*-mediated cell death in this heterologous system (Houterman *et al.*, 2009). The absence of cell death is not due to a lack of Six5 accumulation as immunoblotting showed that TAP-tagged Six5 is detectable in agroinfiltrated *N. benthamiana* leaves (data not shown). Since Avr2 and its variant Avr2^{R45H} both interact with Six5 in yeast, and this interaction could be required for cell death we also co-expressed Δsp*SIX5* and Δsp*AVR2* or Δsp*AVR2* ^{R45H} together with the *I-2*. As shown in Figure 11B and 11C co-expression of these three genes did not influence cell death. Based on these observations, we can conclude that Six5 does not trigger *I-2*-mediated cell death.

Discussion

The *Fusarium*-tomato system is a well-established model to study the interaction between a xylem-colonizing fungus and a plant. Upon infection the fungus secretes many enzymes and

many small proteins into the xylem sap of its host plant. A role in pathogenicity has been shown for three of these small proteins (Takken and Rep, 2010) and we here show that also Six5 is required for full virulence. Deletion of *SIX5* in Fol007 (WT) compromises pathogenicity on tomato seedlings (Figure 1) as well as on older plants (data not shown). Deletion of *SIX5* in race 1 strain Fol004 reduces virulence to a similar degree (data not shown).

A combined function for Six5 and Avr2 for infection of tomato is also suggested by their conservation in *Fol*. Up to now eleven Six proteins have been identified in the xylem sap of *Fol* infected tomato plants (Houterman *et al*., 2007; Lievens *et al*., 2009). Many of these *SIX* genes have homologs in other *Fusarium* species. For instance, *AVR3*, *SIX4*, *SIX8* and *SIX9* homologs are present in the genome of Arabidopsis-infecting *Fusarium oxysporum* isolate Fo5176 (Thatcher *et al*., 2012), a *SIX6* homolog is present in *F. oxysporum* f.sp. *melonis*, *radices-cucumerinum*, and *vasinfectum*, and a *SIX7* homolog in *F. oxysporum* f.sp. *lilii* (Chakrabarti *et al*., 2011; Lievens *et al*., 2009). *SIX5* and *AVR2* appear to be unique for *F. oxysporum* f.sp. *lycopersici* isolates and can be used as markers for *Fol* identification (Lievens *et al*., 2009). Both genes are located at the same location on chromosome 14 and share a promoter region. In yeast and humans, gene-pairs under the control of a bi-directional promoter are prone to be related by function (Liu *et al*., 2011). Expression of both genes is controlled by the SGE1 (*SIX* gene expression 1) transcription factor and deletion of *SGE1* abolishes pathogenicity on tomato as well as expression of *SIX* genes including *AVR2* and *SIX5* (Michielse *et al*., 2009). Besides being co-expressed, an interaction between the gene products was found in the yeast two-hybrid system. However, co-immunoprecipication (Co-IP) failed to verify this interaction *in planta* (data not shown). Possibly, the *in planta* interaction is transient or too weak to survive the purification and washing steps. An interaction between these proteins in the xylem sap might be important for the stabilisation of Six5 as in xylem sap of the Δ*AVR2* strain-inoculated plants Six5 accumulation is reduced (Figure 7 and 8). Stabilisation of Six5 by Avr2 could explain why expression of GFP-tagged *SIX5* through agroinfiltration in *N. benthamiana* leaves did not result in accumulation of Six5 in amounts detectable by antibodies targeted against GFP or Six5 (data not shown). Surprisingly, TAP-tagged Six5 was readily detectable in agroinfiltrated *N. benthamiana* leaves and Six5 tagged with Gal4 activation domain also was detected in yeast extracts, suggesting that TAP tag or Gal4 activation domain could stabilize Six5 obviating the need for Avr2. An alternative explanation for the reduced amount of Six5 in the Δ*AVR2* strain-inoculated tomato is that Avr2 directly, or indirectly, affects specific tomato proteins that mediate the turnover of Six5 during infection. Analysis of the xylem sap proteome to identify proteins whose accumulation differs between WT and the Δ*AVR2* strain-inoculated plants could provide support for the later hypothesis and could reveal identify of these proteins.

Both *SIX5* and *AVR2* are required to trigger *I-2* mediated resistance in the *Fol*-tomato interaction (Figure 3). This observation is consistent with a model in which *I-2* is not activated only by Avr2 but rather by the Six5-Avr2 effector pair. In this model both effectors are required and sufficient to trigger resistance in an *I-2* plant. To our knowledge this is the first report of an effector pair that is required to trigger R protein activation. This interaction constitutes a new variant of the gene-for-gene model in that the pathogen-encoded locus actually consists of a gene-pair rather than a single gene. Avoidance of *I-2* mediated recognition by race 3 isolates is correlated with specific point mutations in Avr2. Apparently, these mutations allow the Avr2 protein to retain its virulence function while shedding its *I-2* activating activity. We did not find polymorphisms in Six5 in race 3 isolates in our *Fol* collection, which indicates that selection for breaking of *I-2* mediated preferably occurs on *AVR2*. Possibly, mutations in *SIX5* that allow evasion of recognition also compromise its virulence function.

While the Six5-Avr2 pair is required to induce *Fol* resistance in tomato, *AVR2* alone is sufficient to induce *I-2*-mediated cell death upon expression in either *N. benthamiana* leaves or in tomato plants (Houterman *et al*., 2009). Expression of *SIX5* using the same expression vectors did not trigger *I-2*-mediated cell death (Figure 11). This raises the question why Six5 is required for *I-2*-mediated resistance in the *Fol*-tomato interaction, even though it is not required for activation of *I-2* mediated cell death and also not for accumulation of Avr2 in xylem sap. One possibility is that the observed cell death caused by *AVR2* in *N. benthamiana* leaves and in PVX-infected tomato plants is due to overexpression of *AVR2*, obviating the requirement for Six5 in *I-2* activation. Alternatively, cell death and resistance might be independent processes, the first one only being observed upon *AVR2* overexpression while the second one represent the genuine resistance response upon recognition of the Avr2 - Six5 pair. Support for this hypothesis is that cell death is not typically observed in an incompatible interaction between *Fol* and tomato. *I-2* is expressed in xylem parenchyma cells adjacent to the vessel elements (Mes *et al*., 2000), and also the typical defence responses are found in those cells. These responses involve the accumulation of phenolic compounds, deposition of callose and gums in the infected vessels, and the formation of tyloses (outgrowth of xylem contact cells) but no or little cell death (Beckman, 2000; Takken and Rep, 2010). Yet, these plants are resistant to *Fol* showing that resistance to Fusarium wilt does not require a cell death response. In other systems, NB-LRR-mediated cell death and resistance can also be uncoupled, as for instance shown for RPS4 conferring resistance against *Pseudomonas syringae* (Heidrich *et al*., 2011). A nuclear localization of this protein is required for AvrRps4 mediated resistance whereas a cytoplasmic location induces cell death but no *Pseudomonas* resistance. Likewise, for Mla10 cell death and resistance can be uncoupled by translocating the protein from the cytoplasm to the nucleus (Bai *et al*., 2012). By analogy, we envisage the intriguing possibility that Avr2 is recognised

in the nucleus to trigger cell death (described in chapter 4), but requires Six5 in the cytosol to trigger resistance. Future studies should put this model to the test.

Materials and methods

Fungal strains, plant materials and bioassay on tomato

Fusarium oxysporum f.sp. *lycopersici* strain 004 (Fol004), 007 （Fol007) and 029 (Fol029) were used (Houterman *et al.*, 2009). Fol007 and Fol004 were used as recipient strains for fungal transformations. Tomato (*Solanum lycopersicum*) cultivar C32, which is susceptible to all *Fol* isolates (Kroon and Elgersma, 1993) and 90E341F (Stall and Walter, 1965), which is resistant to Fol007 (race 2), were used for bioassays. For bioassays, *Fol* was inoculated in liquid medium (100 mM KNO_3, 3% sucrose and 0.17% Yeast Nitrogen Base without amino acids and ammonium sulfate (Difco)) and spores were harvested after three-five days of growth at 25 ºC with shaking at 175 rpm. After washing the spores with sterilized water they were diluted to 10^7 spore/ml. Ten-day-old tomato seedlings of plant cultivars C32 and 90E341F were used for *Fol* inoculations using to the root dip method (Mes *et al.*, 1999). Disease index and plant weight were scored and statistical analysis was performed as described before (Houterman *et al.*, 2009).

Generation of the *Fol SIX5* knockout and its complementation

Gene knockout strains were generated using homologous recombination to replace the *SIX5* gene with a hygromycin resistance cassette. To generate the hygromycin *SIX5* knockout construct, up and downstream regions of *SIX5* open reading frame were amplified by PCR using Fol007 genomic DNA as template with respectively primer combination FP1505 and FP1506 or FP1507 and FP1508 (Table S1). Subsequently, the 809 bp upstream region of *SIX5,* flanked with *Pac*I and *Kpn*I restriction sites, was cloned in front of the hygromycin resistance cassette present in binary vector pRW2h (Houterman *et al.*, 2008). Then, 979 bp downstream sequence of *SIX5,* flanked by *Xba*I and *Bss*HII restriction sites, was introduced behind the hygromycin gene. The obtained plasmid, pRW2hΔ*SIX5,* was transformed into *Agrobacterium* EHA105 to use for Agrobacterium tumefaciens-mediated *Fol* transformation according to the method described by Rep *et al.* (Rep *et al.*, 2004). Transformants capable of growing on 100 µg mL^{-1} hygromycin (Duchefa) were checked by PCR for the absence of the *SIX5* gene using primer pair FP1488/FP1489 and presence of the right and left border part of the construct in combination with a primer just outside the flanking sequences used for pRW2hΔ*SIX5* using primer pairs FP745/FP2281 (right border) and FP659/FP2282 (left border).

For complementation of Δ*SIX5*, a 1826 bp fragment encompassing the complete open reading frame of *SIX5* including 1049 bp upstream and 271 bp downstream sequence of *SIX5* was amplified by PCR from genomic DNA from Fol007 using primers FP1725 and FP1726. The primers added *Xba*I and *Pst*I restriction sites to the PCR product. The obtained PCR fragment was sub-cloned into binary vector pRW1p to generate pRW1Six5Com. The complemented Δ*SIX5* strain was generated by Agrobacterium-mediated *Fol* transformation using pRW1Six5Com as described above. Transformants were selected on 100 µg mL^{-1} zeocin (InvivoGen, http://www.invivogen.com) with 0.1 M Tris pH 8.0 added using the phleomycin resistance cassette in pRW1p (Houterman *et al.*, 2008). The presence of *SIX5* in the zeocin resistant transformants was verified by PCR with primer sets FP1505/FP1957 (promoter region) and FP1488/FP1726 (SIX5 ORF and terminator).

Vector construction

A truncated *SIX5* ORF lacking the signal peptide coding sequence was generated using primer combination FP2701 and FP2164 with template of *Fol*-tomato cDNA library (de la Fuente van Bentem *et al.*, 2005) and gateway *attB* linkers were added via PCR using primers FP872 and FP873. The obtained PCR products were introduced into entry clone pDONR207 (Invitrogen, http://www.invitrogen.com/) using the Gateway protocol described by the manufacturer (Invitrogen). Next, the obtained pENTR207::Δsp*SIX5* plasmid was sub-cloned into the binary vector CTAPi (Rohila *et al.*, 2004) using the Gateway protocol (Invitrogen). In order to create tagged constructs, the stop codon of *SIX5* was replaced by PCR using reverse primer FP2203. Construction of binary CTAPi vectors carrying *AVR2* has been described previously (Houterman *et al.*, 2009).

To generate the yeast-two hybrid constructs, the *AVR2* and its variant $AVR2^{R/H}$ ORF, lacking the sequence encoding the signal peptide, flanked with *Nco*I and *Eco*RI restriction sites were obtained via PCR with primer FP1873 and FP1874 using the *AVR2* and $AVR2^{R/H}$ gene in CTAPi as the template (Houterman *et al.*, 2009). The obtained products were cloned into the pAS2-1 (Clontech) vector with the corresponding restriction sites, respectively. *SIX5* lacking signal peptide coding sequence was amplified from the *Fol*-tomato cDNA library with primer set FP3446 and FP3447, and cloned into the pACT2 (Clontech) vector between *Nco*I and *Eco*RI sites.

For antigen production an *AVR3* gene fragment lacking the signal peptide coding sequence was amplified with primer pair FP2297/FP2298. Subsequently, gateway *attB* linkers were added via PCR using primers FP872 and FP873. The amplified DNA fragment was introduced into entry clone pDONR207 (Invitrogen, http://www.invitrogen.com/) based on the Gateway protocol (Invitrogen) resulting in generation of pENTR207::Δsp*AVR3* plasmid. Next, entry clones pENTR207::Δsp*AVR2* (Houterman *et al.*, 2009), pENTR207*::*Δsp*SIX5* and pENTR207::Δsp*AVR3* were used in an LR reaction to clone the entry genes into the destination gateway vector pGEX-KG-GW (Dhonukshe *et al.*, 2010) according to the Gateway protocol (Invitrogen). The resulting constructs pGEX-KG::Δsp*AVR2,* pGEX-KG::Δsp*AVR3* and pGEX-KG::Δsp*SIX5* encode proteins that carry an N-terminal GST tag.

Binary vectors to express the *SIX* genes in *N. benthamiana* for verification of antibody specificity were generated via an LR reaction (Invitrogen) of the pENTR207::Δsp*AVR2* (Houterman *et al.*, 2009) and pENTR207*::*Δsp*SIX5* into the gateway compatible binary vector pGWB451. Δsp*AVR3,* without its prodomain, was amplified from pENTR207::Δsp*AVR3* using primer pair FP2646 and FP2578. The PCR product was digested with *Xba*I and *Bam*HI and cloned in the same sites of SLD3104 (a gift from Dr. Wladimir Tameling, Wageningen university, Wageningen, the Netherlands) as a translational fusion with a HA-SBP tag. The resulting constructs pGWB451::Δsp*AVR2* pWB451*::*Δsp*SIX5* and SLD3104::Δsp*AVR3* were transformed to *A. tumefaciens* GV3010 as described below. All PCR primers were purchased from MWG (http://www.mwg-biotech.com) and all clones were verified by sequencing.

Agrobacterium-mediated transient transformation of *Nicotiana benthamiana* leaves

Agrobacterium-mediated transient transformation of *Nicotiana benthamiana* was performed essentially as described in (Ma *et al.*, 2012). For immunoblotting *A. tumefaciens* GV3101 was infiltrated at an OD_{600} of 1.0. Thirty-six hour after infiltration leaves were harvested, frozen in liquid nitrogen, and used for protein extraction. To visualise cell death, *A. tumefaciens* GV3101 was

infiltrated using an OD_{600} of 0.2 (*I-2* construct) or 0.5 (*AVR2* or *SIX5* construct), respectively. Three days after infiltration the leaves were photographed and cell death was scored.

Trypan blue staining

Agroinfiltrated leaves 3 days post infiltration were collected and boiled for 5 min in a 1:1 mixture of ethanol and 0.33 mg mL^{-1} trypan blue in lactophenol. Leaves were destained overnight in 2.5 g mL^{-1} chloral hydrate in water.

RNA isolation and RT-PCR analysis

Ten-day-old C32 or 90E341F tomato seedlings were inoculated with water, Fol007, Fol∆*AVR2*, Fol∆*SIX5*, Fol∆*AVR2*+*AVR2* and Fol∆*SIX5*+*SIX5*, respectively with the root dip method and then potted in vermiculite (Agra-vermiculite, Eveleens, Aalsmeer, The Netherlands). Extra nutrients (NPK 20:10:20 final concentration 1 g L^{-1}, Peters Professional, Heerlen, The Netherlands) were applied three times per week during growth. Ten days after inoculation, the roots were cut below the hypocotyls, rinsed twice with water and dried with tissues. The root samples were ground in liquid nitrogen. Total RNA from the samples was extracted with TRIzol LS reagent (Invitrogen) and subsequently purified with RNeasy Mini kit (Qiagen). DNA was removed by on-column treatment with RNase-free DNase (Qiagen). cDNA was synthesized using the M-MulV reverse transcriptase RNase H minus kit (Fermentas, Thermo Scientific). The primer combinations FP962/FP963 and FP1993/FP1994 were used to amplify the *AVR2* and *SIX5* genes from cDNA, respectively. Meanwhile, the *FEM1* gene was amplified using primer set FP157/FP158 as an internal control.

Xylem sap collection and SDS-PAGE

Fol004, Fol029, Fol007, Δ*AVR2* and Δ*SIX5* were used for tomato inoculation. Four-week-old tomato plants C32 were inoculated, after removing part of the root system, with a *Fol* spore suspension (5×10^6 spores mL^{-1}) or with water as a negative control, and potted. Fourteen days post inoculation, tomato plants were photographed and xylem sap was collected as described (Krasikov *et al.*, 2011; Rep *et al.*, 2002). Briefly, stems were cut below the second true leaf and the plant was placed in a horizontal position. Then, for minimal 6 h sap bleeding from the cut surface was collected in tubes placed on ice. The collected xylem sap was stored at -20 ºC freezers.

For label-free protein quantification 25 plants per inoculum were inoculated with Fol007, Fol∆*AVR2*, Fol∆*SIX5* or water. Xylem sap was isolated as described above from four independent biological replicates. A fraction of the sap was used for immunoblotting, the remainder was concentrated with a Centricon plus-70 (Millipore) unit to a final volume of 200-300 µl. The protein concentration was determined with the bicinchoninic acid method (Sigma). After trichloroacetic acid/aceton precipitation protein isolated from inoculated plants with water, Fol007, Fol∆*AVR2* and Fol∆*SIX5*, respectively was dissolved in sample buffer at equal concentration (1.5 µg/µl) and 30 µl per sample was loaded on the SDS-PAGE. SDS–PAGE was performed with Hoefer Mighty Small SE250 minigel equipment (Amersham Biosciences, AB, Uppsala). After a short run, the Coommassie PageBlue™ (Fermentas) was used to visualize the proteins in the SDS-PAGE.

Mass Spectrometry and label free Quantitative proteomics

For each xylem sap sample one gel slice containing all proteins was cut from the coommassie-stained gel. In-gel digestion was performed as described by Rep *et al.* (Rep *et al.*, 2002). The peptides obtained after this digestion were analyzed by nanoLC-MS/MS as described by Lu, *et al* (Lu *et al.*, 2011). Raw data from the LTQ-Orbitrap were analyzed with MaxQuant software (Cox and Mann,

2008; Hubner *et al.*, 2010) to identify the proteins and allow label-free relative quantification. Default MaxQuant 1.1.36 settings were used according to the description by Peng, *et al* (Peng *et al.*, 2012). The *Fol* protein database used for the analysis was obtained from Fusarium Comparative Genome website (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html) and supplemented by adding the sequences of known SIX proteins that are not annotated in the public database. To identify the tomato proteins, the SGN tomato protein database ITAG2 version 3(ftp://ftp.solgenomics.net/../../proteins/protein_predictions_from_unigenes/single_species_assemblies/Solanum_lycopersicum/) was used. Besides these databases, a "contaminant" database was used that contains proteins such as trypsin and human keratins (Peng *et al.*, 2012). Bioinformatics analysis of the MaxQuant workflow and the statistical analysis of the abundances of the identified proteins were performed using Perseus (available at www.MaxQuant.org) (Hubner *et al.*, 2010).

Yeast two-hybrid assays

The matchmaker GAL4 two-hybrid system and yeast strain PJ694a were used for analyzing protein interactions. Yeast transformation was performed using lithium-acetate and polyethylene glycol 3350 as described before (Gietz and Woods, 2002). Eight colonies were picked and transferred from the MM-WL plates, lacking Trp and Leu, to the fresh MM-WL and MM-HAWL plates lacking Trp, Leu, His and Ade, to let them incubate for 7 days at 30°C. Next, one colony per combination was used to inoculate 1 mL MM-WL culture. After 36 h growth, certain amount of cells were spin down and resuspended in 25 µl 0.9 % NaCl to OD_{600}=1. A 10x dilution range was made to $OD_{600}=1 \times 10^{-2}$ and 6 µl per combination was spotted on MM-WL, MM-AWL, and MM-HWL plates. After 5 days incubation at 30 °C, the plates were checked for growth and photographed.

For checking the expression of bait and prey proteins, the rapid protein extraction method from yeast was used (Horvath and Riezman, 1994). Briefly, the transformed yeast grown for 5 days in MM-WL plates was scraped using the bacteriological loop and suspended in 30 µl the optimized protein sample buffer (60mMM Tris pH=6.8, 10% glycerol, 2% SDS, 5% (v/v) 2-mercaptoethanol and 0.0025% (w/v) bromophenol blue). Cells were boiled for 5 minutes, and then centrifuged for 5 minutes. The supernatants were used for loading on the SDS-PAGE gel.

Protein extraction and western blotting

Protein extraction from agro-infiltrated leaves was performed as described before (Ma *et al.*, 2012). Briefly, frozen leaves were ground in liquid nitrogen and suspended in 2 ml protein extraction buffer per gram of tissue [25 mm Tris pH 8, 1 mm EDTA, 150 mm NaCl, 5 mm 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*** at 4°C for 10 min, and the supernatant was passed over four layers of Miracloth (http://www.calbiochem.com/miracloth) to obtain a total protein lysate. Samples were mixed with Laemmli sample buffer, and equal amounts of total protein 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 (PBS, 0.1 % Tween 20). For validation of polyclonal antibodies proteins were extracted from *N. benthamiana* leaves expressing Avr2-GFP, SIX5-GFP and Avr3-HASBP with a buffer composed of 9.5 M urea, 100 mM Tris pH 6.8, 2% SDS, 5 mM DTT. Antisera of Avr3 (Six1), Avr2 and Six5 were tested at a dilution of 1:500. The secondary antibody goat-anti-rabbit conjugated with horseradish peroxidase (Pierce, 31210) was used at a 1:5.000 dilution. The signal was visualized by ECL using BioMax MR film (Kodak, http://www.kodak.com).

For immunoblotting xylem sap, 20 µl xylem sap was loaded on 15% TRIS-Tricine gel (Schagger and von Jagow, 1987). After electrophoresis, the proteins were blotted onto PVDF membranes using semi-dry blotting. Skimmed milk powder (5%) was used as blocking agent. Anti-Avr2 antibody was used at a 1:10.000 dilution, anti-Avr3 and anti-Six5 at a 1:5.000 dilution.

For immunoblotting to check yeast proteins, 30 µl resulting yeast protein fractions (supernatant) were loaded on SDS–PAGE gels. The blot with the bait was probed using anti-Avr2 antibody at 1:5000 dilution and for the prey using horseradish peroxidase-conjugated anti-HA antibody (monoclonal 12CA5; Roche) at a 1:3000 dilution. The secondary antibody goat-anti-rat conjugated with horseradish peroxidase (Pierce, 31470) was used at 1:5.000 dilution for detection of HA.

Co-immunoprecipication

For co-IP experiments, total proteins were extracted from *N. benthamiana* leaves 36 h after infiltrating *A. tumefaciens* GV3101 containing either pBIN61::Δsp*AVR2*-HASBP or CTAPi::Δsp*SIX5*-TAP or a mixture of both *A. tumefaciens* strains. Extracts were spun for 10 min at 12,000g, and 1 mL supernatant was added to 100 µL bed volume of Streptavidin Sepharose High Performance beads (GE Healthcare). Protein extracts were incubated in a rotator for 3 h at 4°C, and washed four times with immunoprecipitation buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, and 5 mM DTT, and 0.15% Nonidet P-40). Elution was performed twice with two bed volumes of wash buffer containing 4 mM D-biotin (Sigma-Aldrich). Elution fractions were pooled and precipitated with trichloroacetic acid. Pellets were washed with 100% acetone at –20 °C. A portion of the supernatant was reserved as input sample. Immunoprecipicated samples and input samples were resuspended in 1x SDS-PAGE loading buffer and loaded on 12% SDS-PAGE gels. Next, the gels were subjected to immunoblotting using anti-HA peroxidase (clone 3F10; Roche), and anti-TAP (PAP, P129, Sigma).

Polyclonal antibody generation

Expression vectors pGEX-KG::Δsp*AVR2,* pGEX-KG::Δsp*SIX1* and pGEX-KG::Δsp*SIX5* were transformed to *E.coli* BL21 (DE3) and Six/Avr proteins were expressed after adding 1mM IPTG at an OD_{600} of 0.8 and subsequent growth for 3 hour at 18 °C while shaking at 180 rpm. Cells were harvested by centrifugation at 5,000 g and 4 °C for 15 min and resuspended in lysis buffer (1mg/mL lysozyme, 0.2 % triton X-100 in PBS, pH=7.4) supplemented with protease inhibitor (complete, Roche). The cell suspension was incubated at 4°C for 20 min with gentle agitation and sonicated 4 x 20 sec. Subsequently, the suspension was centrifuged at 13,000 g and 4 °C for 30 min. The cleared *E. coli* homogenate was incubated with PBS-equilibrated glutathione-sepharose (GE healthcare) for one hour at 4°C with gentle rotation. Washing steps with PBS were performed following the manufacturer's instructions, and the recombinant protein was eluted from the glutathione-sepharose with 20 mM reduced glutathione, 150 mM NaCl, 50 mM Tris-HCl pH 8.5.

One mg of purified Avr2, Avr3 and Six5 protein with GST tag was used for immunization of two rabbits according to the manufacture's high-speed protocol (BioGenes). Only rabbits were used of which their pre-immune sera did not cross-react with plant proteins in a range of 10-30 kDa that were isolated from *N. benthamiana, Lycopersicon esculentum* and *Arabidopsis thaliana* leaves.

Supplementary data

Table S1 Primers used in this study

Primer name	Sequences
FP1505	AAATTAATTAACCAGCTAAGCTAGCGGTTAC
FP1506	AAAGGTACCAGACCATGTTAGCAAACACAC
FP1507	AAATCTAGAGTCTACAGCTTAGGATGATTA
FP1508	AAAGCGCGCGACCTGTCGGGTAGTGATTG
FP1488	ACACGCTCTACTACTCTTCA
FP1489	GAAAACCTCAACGCGGCAAA
FP745	GCATGTTTCTTCCTTGAACTCTC
FP2281	CATATAGCCATGTCTGGAGG
FP659	TAGAGATCATGCTATATCTC
FP2282	GTACAAAGCTAAAGTGCAAT
FP1725	AAATCTAGACATTATACCGTTCGGTCCTT
FP1726	AAACTGCAGTCTCACATCCTATCGCGACA
FP1957	GTCAGCAAACCAATATGCTG
FP2578	AAAAGGATCCTTAGTGTGGGCTGGTATATCC
FP2701	AAAAAGCAGGCTGGATGAGGGATCATCAGTACTGTGCT
FP2203	AGAAAGCTGGGTCGCCGCATCACAATAGATAAC
FP2164	AGAAAGCTGGGTCCTAGGCCGCATCACAATAGAT
FP3702	GTAGGCGCGCCAGAACACGGGGGACTCTAGA
FP3703	GTAATAAGAGCGGCCGCACGATCGGGGAAATTCGAGCTC
FP1873	AAACCATGGAAGATGCCGATTCATC
FP1874	AAAGAATTCAATCCTCTGAGATAGTAAG
FP3446	CGCCATGGCAAGGGATCATCAGTACTGTGCTTGC
FP3447	CGAGAATTCCTAGGCCGCATCACAATAGATAACG
FP962	TGAGCGGGCTGGCAATTC
FP963	CAATCCTCTGAGATAGTAAG
FP1993	GCGCTTCGAGTACATCTCTG
FP1994	CTAGGCCGCATCACAATAGA
FP157	ATGAAGTACACTCTCGCTACC
FP158	GGTGAAAGTGAAAGAGTCACC
FP2297	AAAAGCAGGCTCCATGGCGCCCTATAGCATG
FP2298	AGAAAGCTGGGTCTTAGTGTGGGCTGGTATATCCA
FP1405	GTTGGTCAAGACCAATGCGGAG
FP1406	TGCACAGGGTGTCACGTTGC
FP2646	AAAATCTAGAATGGAGCCTTTCGGGGAGGAG
FP872	GGGGACAAGTTTGTACAAAAAAGCAGGCT
FP873	GGGGACCACTTTGTACAAGAAAGCTGGGT

Acknowledgement

Many tanks to Fang Xu for assisting the xylem sap isolation and to Ludek Tikovsky, Harrold Lemereis and Thijs Hendrix for plant care and greenhouse management.

References

Bai S, Liu J, Chang C, Zhang L, Maekawa T, Wang Q, Xiao W, Liu Y, Chai J, Takken FWL, Schulze-Lefert P, Shen Q-H (2012) Structure-function analyses of barley NLR immune receptor MLA10 reveal its subcellular specific activity in cell death signaling and disease resistance. *PLoS Pathog* **In press**

Beckman CH (2000) Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? *Physiol Mol Plant P* **57:** 101-110.

Chakrabarti A, Rep M, Wang B, Ashton A, Dodds P, Ellis J (2011) Variation in potential effector genes distinguishing Australian and non-Australian isolates of the cotton wilt pathogen *Fusarium oxysporum* f.sp *vasinfectum*. *Plant Pathol* **60:** 232-243.

Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* **26:** 1367-1372.

de la Fuente van Bentem S, Vossen JH, de Vries KJ, van Wees S, Tameling WI, Dekker HL, de Koster CG, Haring MA, Takken FL, Cornelissen BJ (2005) Heat shock protein 90 and its co-chaperone protein phosphatase 5 interact with distinct regions of the tomato I-2 disease resistance protein. *Plant J* **43:** 284-298.

Dhonukshe P, Huang F, Galvan-Ampudia CS, Mahonen AP, Kleine-Vehn J, Xu JA, Quint A, Prasad K, Friml J, Scheres B, Offringa R (2010) Plasma membrane-bound AGC3 kinases phosphorylate PIN auxin carriers at TPRXS(N/S) motifs to direct apical PIN recycling. *Development* **137:** 3245-3255.

Dodds PN, Rathjen JP (2010) Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat Rev Genet* **11:** 539-548.

Gietz RD, Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* **350:** 87-96.

Heidrich K, Wirthmueller L, Tasset C, Pouzet C, Deslandes L, Parker JE (2011) Arabidopsis EDS1 Connects Pathogen Effector Recognition to Cell Compartment-Specific Immune Responses. *Science* **334:** 1401-1404.

Horvath A, Riezman H (1994) Rapid protein extraction from *Saccharomyces cerevisiae*. *Yeast* **10:** 1305-1310.

Houterman PM, Cornelissen BJ, Rep M (2008) Suppression of plant resistance gene-based immunity by a fungal effector. *PLoS Pathog* **4:** e1000061.

Houterman PM, Ma L, van Ooijen G, de Vroomen MJ, Cornelissen BJC, Takken FLW, Rep M (2009) The effector protein Avr2 of the xylem-colonizing fungus *Fusarium oxysporum* activates the tomato resistance protein I-2 intracellularly. *Plant Journal* **58:** 970-978.

Houterman PM, Speijer D, Dekker HL, CG DEK, Cornelissen BJ, Rep M (2007) The mixed xylem sap proteome of *Fusarium oxysporum*-infected tomato plants. *Mol Plant Pathol* **8:** 215-221.

Hubner NC, Bird AW, Cox J, Splettstoesser B, Bandilla P, Poser I, Hyman A, Mann M (2010) Quantitative proteomics combined with BAC TransgeneOmics reveals in vivo protein interactions. *J Cell Biol* **189:** 739-754.

Jones JD, Dangl JL (2006) The plant immune system. *Nature* **444:** 323-329.

Kensche PR, Oti M, Dutilh BE, Huynen MA (2008) Conservation of divergent transcription in fungi. *Trends Genet* **24:** 207-211.

Krasikov V, Dekker HL, Rep M, Takken FL (2011) The tomato xylem sap protein XSP10 is required for full susceptibility to Fusarium wilt disease. *J Exp Bot* **62:** 963-973.

Kroon BAM, Elgersma DM (1993) Interactions between Race-2 of *Fusarium oxysporum* f. sp. *Lycopersici* and near-isogenic resistant and susceptible lines of intact plants or callus of Tomato. *J Phytopathol* **137:** 1-9.

Lievens B, Houterman PM, Rep M (2009) Effector gene screening allows unambiguous identification of *Fusarium oxysporum* f. sp. *lycopersici* races and discrimination from other formae speciales. *FEMS Microbiol Lett* **300:** 201-215.

Liu BC, Chen JJ, Shen BR (2011) Genome-wide analysis of the transcription factor binding preference of human bi-directional promoters and functional annotation of related gene pairs. *Bmc Syst Biol* **5**

Lu J, Boeren S, de Vries SC, van Valenberg HJF, Vervoort J, Hettinga K (2011) Filter-aided sample preparation with dimethyl labeling to identify and quantify milk fat globule membrane proteins. *J Proteomics* **75:** 34-43.

Ma L, Lukasik E, Gawehns F, Takken FL (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.

Ma LJ, van der Does HC, Borkovich KA, Coleman JJ, Daboussi MJ, Di Pietro A, Dufresne M, Freitag M, Grabherr M, Henrissat B, Houterman PM, Kang S, Shim WB, Woloshuk C, Xie X, Xu JR, Antoniw J, Baker SE, Bluhm BH, Breakspear A, Brown DW, Butchko RA, Chapman S, Coulson R, Coutinho PM, Danchin EG, Diener A, Gale LR, Gardiner DM, Goff S, Hammond-Kosack KE, Hilburn K, Hua-Van A, Jonkers W, Kazan K, Kodira CD, Koehrsen M, Kumar L, Lee YH, Li L, Manners JM, Miranda-Saavedra D, Mukherjee M, Park G, Park J, Park SY, Proctor RH, Regev A, Ruiz-Roldan MC, Sain D, Sakthikumar S, Sykes S, Schwartz DC, Turgeon BG, Wapinski I, Yoder O, Young S, Zeng Q, Zhou S, Galagan J, Cuomo CA, Kistler HC, Rep M (2010) Comparative genomics reveals mobile pathogenicity chromosomes in Fusarium. *Nature* **464:** 367-373.

Mes JJ, van Doorn AA, Wijbrandi J, Simons G, Cornelissen BJ, Haring MA (2000) Expression of the Fusarium resistance gene I-2 colocalizes with the site of fungal containment. *Plant J* **23:** 183-193.

Mes JJ, Wit R, Testerink CS, de Groot F, Haring MA, Cornelissen BJ (1999) Loss of avirulence and reduced Ppathogenicity of a Gamma-irradiated mutant of *Fusarium oxysporum* f. sp. *lycopersici*. *Phytopathology* **89:** 1131-1137.

Michielse CB, Rep M (2009) Pathogen profile update: *Fusarium oxysporum*. *Mol Plant Pathol* **10:** 311-324.

Michielse CB, van Wijk R, Reijnen L, Manders EM, Boas S, Olivain C, Alabouvette C, Rep M (2009) The nuclear protein Sge1 of *Fusarium oxysporum* is required for parasitic growth. *PLoS Pathog* **5:** e1000637.

Peng K, van Lent JW, Boeren S, Fang M, Theilmann DA, Erlandson MA, Vlak JM, van Oers MM (2012) Characterization of novel components of the baculovirus per os infectivity factor (PIF) complex. *J Virol* **In press**

Rep M, Dekker HL, Vossen JH, de Boer AD, Houterman PM, Speijer D, Back JW, de Koster CG, Cornelissen BJ (2002) Mass spectrometric identification of isoforms of PR proteins in xylem sap of fungus-infected tomato. *Plant Physiol* **130:** 904-917.

Rep M, Kistler HC (2010) The genomic organization of plant pathogenicity in Fusarium species. *Curr Opin Plant Biol* **13:** 420-426.

Rep M, van der Does HC, Meijer M, van Wijk R, Houterman PM, Dekker HL, de Koster CG, Cornelissen BJ (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 JS, Chen M, Cerny R, Fromm ME (2004) Improved tandem affinity purification tag and methods for isolation of protein heterocomplexes from plants. *Plant J* **38:** 172-181.

Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* **166:** 368-379.

Stall RE, Walter JM (1965) Selection and inheritance of resistance in tomato to isolates of races 1 and 2 of the Fusarium wilt organism. *Phytopathology* **55:** 1213-1215.

Takken F, Rep M (2010) The arms race between tomato and *Fusarium oxysporum*. *Mol Plant Pathol* **11:** 309-314.

Thatcher LF, Gardiner DM, Kazan K, Manners JM (2012) A highly conserved effector in *Fusarium oxysporum* is required for full virulence on Arabidopsis. *Mol Plant Microbe Interact* **25:** 180-190.

Tjamos EC (1989) *Problems and prospects in controlling Verticillium wilt*, Berlin: Springer-Verlag.

van der Does HC, Duyvesteijn RG, Goltstein PM, van Schie CC, Manders EM, Cornelissen BJ, Rep M (2008) Expression of effector gene SIX1 of *Fusarium oxysporum* requires living plant cells. *Fungal Genet Biol* **45:** 1257-1264.

Yang WL, Ng P, Zhao MH, Wong TKF, Yiu SM, Lau YL (2008) Promoter-sharing by different genes in human genome - CPNEI and RBMI2 gene pair as an example. *Bmc Genomics* **9**