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

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Publication date
2012

CHAPTER 3

The effector protein Avr2 of the xylem colonizing fungus *Fusarium oxysporum* activates tomato resistance protein I-2 intracellularly

Abstract

To promote host colonization, many plant-pathogens secrete effector proteins that either suppress or counteract host defences. However, when these effectors are recognized by the host's innate immune system, they trigger resistance rather than promote virulence. Effectors are therefore key-molecules in determining disease susceptibility or resistance. We show here that Avr2, secreted by the vascular wilt fungus *Fusarium oxysporum f. sp. lycopersici* (*Fol*), shows both activities: it is required for full virulence in a susceptible host and also triggers resistance in tomato plants carrying the resistance gene *I-2*. Point mutations in Avr2, causing single amino acid changes, are associated with *I-2*-breaking *Fol* strains. These point mutations prevent recognition by *I-2,* both in tomato and when both genes are co-expressed in leaves of *Nicotiana benthamiana*. *Fol* strains carrying the Avr2 variants are equally virulent, showing that virulence and avirulence functions can be uncoupled. Although Avr2 is secreted into xylem sap when *Fol* colonizes tomato, the Avr2 protein can be recognized intracellularly by I-2, implying uptake by host cells.

This chapter has been published as:

P.M. Houterman*, L. Ma*, G. van Ooijen, M.J. de Vroomen, B.J.C. Cornelissen, F. L.W. Takken and M. Rep (2009) The Plant Journal, Volume 58, 970-978. *Equal contribution

Introduction

Plant pathogenic microbes use small secreted proteins, called effectors, to suppress or evade basal immune responses that would otherwise inhibit host colonization. A second layer of the plant immune system employs surveillance (or resistance) proteins that specifically recognize a subset of these effectors. Recognition occurs either through direct effector binding or indirectly, by their actions in the host (Chisholm *et al.*, 2006; Jones and Dangl, 2006; Tameling and Takken, 2008). Effectors that are recognized by resistance proteins are referred to as avirulence (Avr) proteins. Among the best studied effectors are those that are injected into plant cells by plant pathogenic bacteria through the type III secretion machinery (Buttner and Bonas, 2002). These bacterial effectors target diverse cellular processes such as signal transduction, transcription and vesicle trafficking, and can be detected by intracellular resistance proteins (da Cunha *et al.*, 2007; He *et al.*, 2007; Zhou and Chai, 2008).

Oomycetes do not inject effectors into plant cells, but secrete them into the apoplastic spaces, where they either act to inhibit enzymes or are taken up by plant cells and act inside the host cells on as yet unidentified targets (Birch *et al.*, 2006; Kamoun, 2006; Morgan and Kamoun, 2007). Effectors secreted by fungi can also act either extracellularly or intracellularly (Rep, 2005; Catanzariti *et al.*, 2007). Most fungal effectors identified to date are from leaf-invading pathogens, and effectors that are recognized by intracellular surveillance proteins have only been identified for fungi that form specialized feeding structures called haustoria. These feeding structures project into host cells and are in intimate contact with the plant cell membrane (Catanzariti *et al.*, 2007).

The only specialized root-invading fungus from which effector /avirulence genes have been cloned is the vascular wilt fungus *Fusarium oxysporum*. Host-specific forms (*formae speciales* or *f. sp.*) of *F. oxysporum* can infect a wide variety of plant species resulting in either wilt (due to xylem colonization) or root, bulb or foot-rot (Armstrong and Armstrong, 1981; Tjamos and Beckman, 1989; Di Pietro *et al.*, 2003). *F. oxysporum f. sp. lycopersici (Fol)* causes wilt disease in tomato. Resistant tomato plants carry race-specific resistance genes, called *I* for *Immunity* genes, of which *I*, *I-2* and *I-3* have been introgressed in commercial varieties (Huang and Lindhout, 1997). Only *I-2* has been cloned; it encodes a classical NBS-LRR type resistance protein (Simons *et al.*, 1998), and is expressed specifically in vascular tissue (Mes *et al.*, 2000). To date, two avirulence proteins of *Fol* have been identified: Avr3 (Six1), which is required for *I-3*-mediated resistance (Rep et al., 2004) and Avr1 (Six4) which is required for *I*-mediated resistance (Houterman et al., 2008). Both proteins also have a function in addition to triggering avirulence; Avr3 has been shown to be required for full virulence (Rep et al., 2005), whereas Avr1 suppresses *I-2* and *I-3* mediated disease resistance (Houterman et al., 2008).

In agricultural settings, *I* has been broken by simple loss of *AVR1*, concomitant with a loss of *I-2* and *I-3* suppression (Houterman et al., 2008). *I-2*-mediated resistance against *Fol* has been overcome in the field by 'race 3' isolates in North America and Australia (Cai *et al.*, 2003; Gale *et al.*, 2003), and more recently in Japan (Kawabe *et al.*, 2005) and Brazil (Reis and Boiteux, 2007).

Here, we show that Six3, a small in xylem secreted protein (Houterman *et al.*, 2007), corresponds to Avr2 and can activate I-2 inside plant cells. Evasion of I-2-mediated recognition by race 3 strains is not associated with *AVR2* gene loss. Instead, each race 3 strain investigated was found to contain a single amino acid change in Avr2 that prevents activation of I-2.

Results

Identification of Avr2

Six3 is one of several small proteins secreted by *Fol* during colonization of tomato xylem vessels that has been identified through a proteomics approach (Houterman *et al.*, 2007). Its intron-less gene is predicted to encode a 15.7 kDa mature protein (after cleavage of the predicted N-terminal signal peptide) without similarity to other known proteins. The protein appears in various positions in 2D gels of xylem sap from *Fol*-infected tomato plants, corresponding to apparent sizes from 11-14 kD, probably as a result of proteolytic processing from the N-terminus (Houterman *et al.*, 2007). To determine the biological function of Six3 for *Fol*, we produced knockout mutants in which the *SIX3* gene was replaced by a hygromycin resistance gene cassette. These deletion mutants showed reduced virulence towards susceptible tomato plants, suggesting that Six3 is required for the development of full disease symptoms (Figure 1). Strikingly, when inoculated on a tomato cultivar carrying the *I-2* resistance gene, the *SIX3* deletion mutants had acquired the ability to cause disease, in contrast to the parental strain (Figure 1a). Re-introduction of the *SIX3* gene into one of the deletion mutants restored avirulence (Figure S1). We therefore conclude that Six3 is the avirulence factor that is recognized by I-2, and henceforth designate Six3 as Avr2.

Evasion of I-2-mediated recognition is associated with point mutations in *AVR2*

In various localities, strains that overcome *I-2*-mediated resistance, called race 3 strains, have emerged (see introduction). To investigate whether the ability to avoid *I-2* mediated recognition is correlated to changes in *AVR2*, we determined the presence of *AVR2,* and its sequence, in a representative set of diverse *Fol* strains (Table 1). Using primers just outside

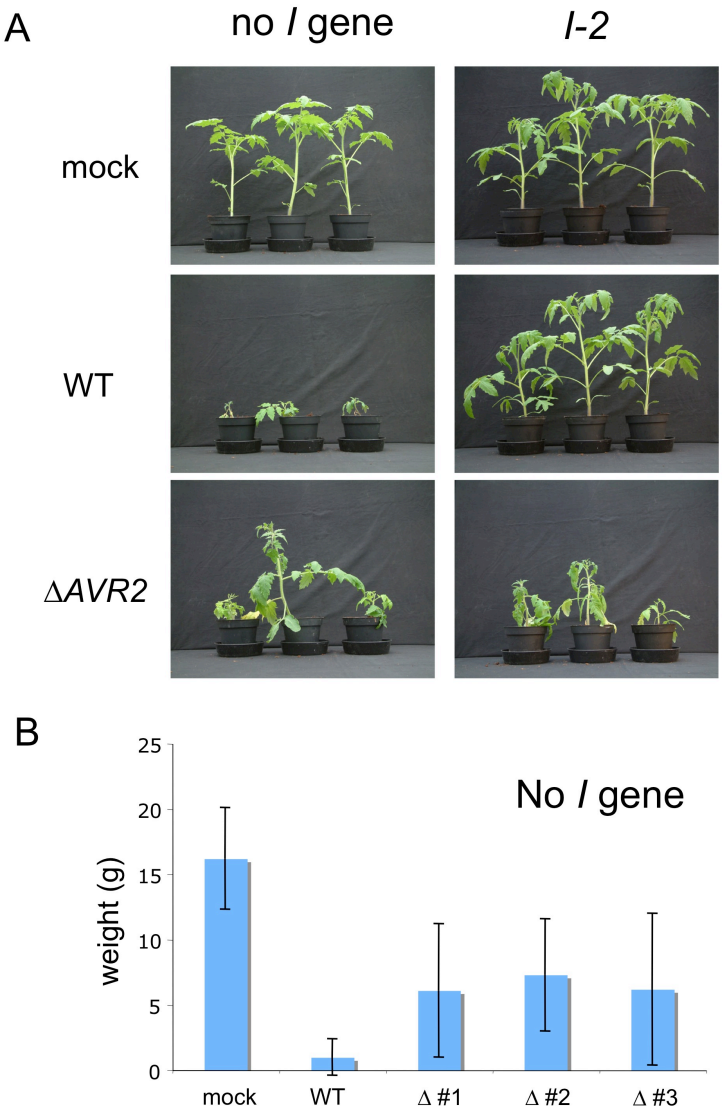


Figure 1. ***I-2*-mediated disease resistance is dependent on the *AVR2* (*SIX3*) gene.** Ten-day-old seedlings of tomato plant lines without resistance genes to *Fol* (no *I* genes) or with only the *I-2* gene (*I-2*) were inoculated with a race 2 strain Fol007 (WT) or a mutant derived from Fol007 in which the *AVR2* gene was deleted (Δ*AVR2*). As a control, both plant lines were also mock-inoculated (mock). **A)** Representative plants 3 weeks after inoculation. **B)** Mean weight above cotyledons of 20 plants without *I* genes after infection with *Fol* strains or mock inoculation (error bars indicate standard deviation). ANOVA revealed that the three independent *AVR2* knock out strains (Δ #1-3) were significantly ($p<0.05$) less virulent than the parent strain Fol007 (WT).

the open reading frame, the *AVR2* coding sequences could be amplified from all tested strains, regardless of race. This observation agrees with earlier findings (van der Does et al., 2008) and implies that the ability to overcome *I-2*-mediated resistance does not involve loss of *AVR2*, which would result in a fitness penalty for the fungus.

Table 1: *Fol* strains for which *AVR2* was sequenced in this study

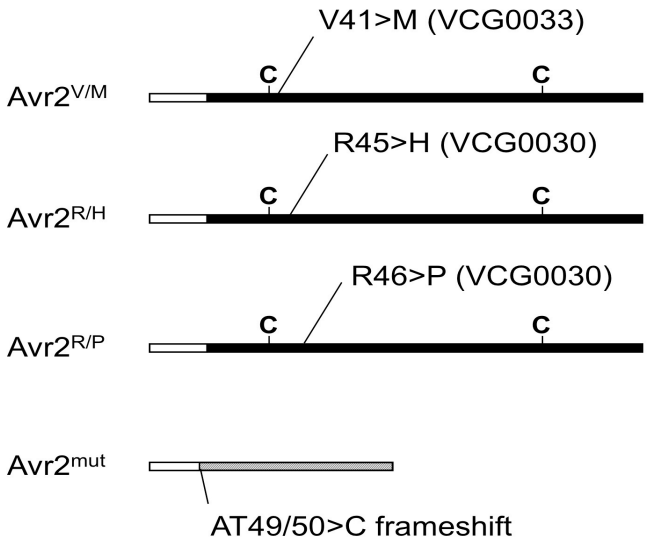
Strain number	Original designation	Origin	Race	VCG[a]	Avr2 mutation
Fol001	WCS801 / E329	Netherlands	1		
Fol004	IPO1530 / B1	Netherlands	1	0030	
Fol008	E79	Netherlands	1		
Fol010	E175	Netherlands	1	0031	
Fol016	BFOL-51	USA, Louisiana	1	0031	
Fol041	MD-S2	USA	1		
Fol002	WCS862 / E241	Netherlands	2	0030	
Fol005	C24 / B2	Netherlands	2		
Fol007	D2	France	2	0030	
Fol017	OSU-451	USA, Ohio	2	0031	
Fol018	LSU-7	USA, Louisiana	2	0032[b]	
Fol026	BRIP14844	Australia	3	0030	R45→H
Fol029	5397	USA, Florida	3	0030	R45→H
Fol035	IPO3		3	0030	R46→P
Fol036		USA, Florida	3	0030	R46→P
Fol038	CA92/95	USA, California	3	0030	R46→P
Fol066	MM25	USA, Arkansas	3	0033	V41→M
Fol067	MM10	USA, Arkansas	3	0033	V41→M

[a]Vegetative compatibility group, corresponds to clonal line.

[b]Originally designated as VCG0032; however, VCG0032 was later found to be part of VCG0030 (Mes et al., 1999a).

All strains in our collection that are avirulent on *I-2* plants were found to contain identical *AVR2* sequences. This high sequence conservation is in line with the close relatedness of the genomic region in which *AVR2* resides between strains of *Fol* (van der Does et al., 2008). In contrast, all *I-2*-breaking (race 3) strains contain a single nucleotide substitution leading to an amino acid change in the Avr2 protein (Figure 2). To date, race 3 strains have emerged in two vegetative compatibility groups (VCGs), which can be considered as independent clonal lines of *F. oxysporum* (Cai et al., 2003). Two mutations in adjacent arginine residues were found in VCG0030: R45→H, corresponding to G134→A, in two strains from Florida and one from Australia, and R46→P, corresponding to G137→C, in a Californian strain and a strain whose origin is unknown to us. In VCG0033, another mutation was found in close proximity to these two arginine residues: V41→M, corresponding to G121→A, present in two strains from Arkansas) (Figure 2). Interestingly, sequencing of the *AVR2* gene in a gamma radiation-induced *I-2*-breaking mutant (Mes *et al.*, 1999b), revealed a frameshift mutation near the 5' end of *AVR2* (AT49/50→C). This mutation in the coding sequence for the signal peptide and hence no Avr2 protein can be

A



B

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Avr2    mrfllliamsmtwvcsiagLPVEDADSSVGQLQGRGNPYCVFPGRRTSSTSFTTSFSTEP   60
V/M     mrfllliamsmtwvcsiagLPVEDADSSVGQLQGRGNPYCMFPGRRTSSTSFTTSFSTEP
R/H     mrfllliamsmtwvcsiagLPVEDADSSVGQLQGRGNPYCVFPGHRTSSTSFTTSFSTEP
R/P     mrfllliamsmtwvcsiagLPVEDADSSVGQLQGRGNPYCVFPGRPTSSTSFTTSFSTEP
                                             *   **

Avr2    LGYARMLHRDPPYERAGNSGLNHRIYERSRVGGLRTVIDVAPPDGHQAIANYEIEVRRIP  120
V/M     LGYARMLHRDPPYERAGNSGLNHRIYERSRVGGLRTVIDVAPPDGHQAIANYEIEVRRIP
R/H     LGYARMLHRDPPYERAGNSGLNHRIYERSRVGGLRTVIDVAPPDGHQAIANYEIEVRRIP
R/P     LGYARMLHRDPPYERAGNSGLNHRIYERSRVGGLRTVIDVAPPDGHQAIANYEIEVRRIP

Avr2    VATPNAAGDCFHTARLSTGSRGPATISWDADASYTYYLTISED                   163
V/M     VATPNAAGDCFHTARLSTGSRGPATISWDADASYTYYLTISED
R/H     VATPNAAGDCFHTARLSTGSRGPATISWDADASYTYYLTISED
R/P     VATPNAAGDCFHTARLSTGSRGPATISWDADASYTYYLTISED
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Figure 2. Each *I-2* breaking (race 3) strain of *Fol* contains a single point mutation in *AVR2* leading to an amino acid replacement. A) Schematic representations of the Avr2 versions found in various *I-2*-breaking strains of *Fol*, showing the position of the predicted signal peptide for secretion (open box), the two cysteine residues (C) that potentially form a disulfide bridge, and the position of the mutation found in various *AVR2* alleles. The VCG numbers in parentheses indicate the clonal line (vegetative compatibility group) to which strains carrying the indicated mutation belong. $Avr2^{mut}$ is the predicted product of the mutated *AVR2* gene in a gamma irradiation-induced *avr2* mutant (Mes *et al.*, 1999b); the dashed area in this product represents an out-of-frame translation ending at a stop codon about halfway along the original open reading frame. **B)** Amino acid sequence alignment of wild-tpe Avr2 with the three versions from race 3 strains (V/M, R/H and R/P), with amino acid replacements in bold. The predicted signal peptide is shown in lower case.

produced by this mutant. In line with this, the mutant showed reduced virulence towards a susceptible tomato line (Mes *et al.*, 1999b), similar to the *AVR2* deletion mutants described above.

The fact that we found only point mutations in AVR2 and no deletions or frameshift mutations in naturally occurring race 3 strains is in line with our observation that *AVR2* is

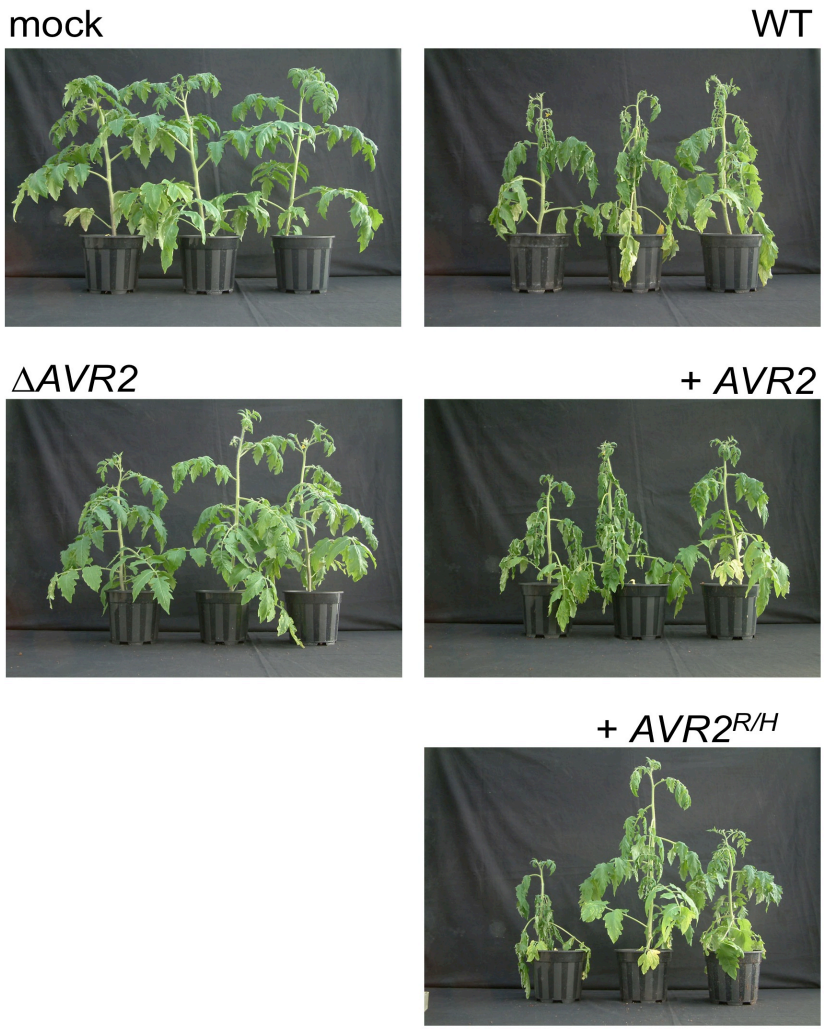

Figure 3. The R45→H mutation does not affect the virulence function of Avr2. Five week old tomato plants without resistance genes to *Fol* (i.e. no *I* genes) were either mock-inoculated (mock) or inoculated with a race 2 isolate Fol007 (WT), a mutant derived from Fol007 in which the *AVR2* gene was deleted (Δ*AVR2*), the Δ*AVR2* strain transformed with the wild-type *AVR2* gene (+*AVR2*), or the Δ*AVR2* strain transformed with the R45→H mutant of *AVR2* from a race 3 strain (+$AVR2^{R/H}$). Loss of *AVR2* reduces virulence on susceptible tomato plants, which can be restored by reintroduction of *AVR2* or $AVR2^{R/H}$. Pictures were taken 14 days after inoculation.

required for full virulence. To confirm that the point mutations in *AVR2* in race 3strains do not affect its virulence function, we transformed the *AVR2* deletion strain (Δ*AVR2*) with both wild-type *AVR2* and one of the mutant versions from a race 3 strain (R45→H). As with deletion of *AVR3* (*SIX1*) (Rep *et al.*, 2005), deletion of *AVR2* results in a more pronounced reduction in virulence towards older plants when compared to seedlings (Figure 3, compare WT with Δ*AVR2*). Reintroduction of either wild-type *AVR2* or $AVR2^{R/H}$ restored virulence, demonstrating directly that the R45→H mutation does not affect the virulence function of

Avr2.

Activation of I-2 by Avr2 in *Nicotiana benthamiana* and tomato

R-gene based resistance against *Fol* operates in the xylem parenchyma cells that surround

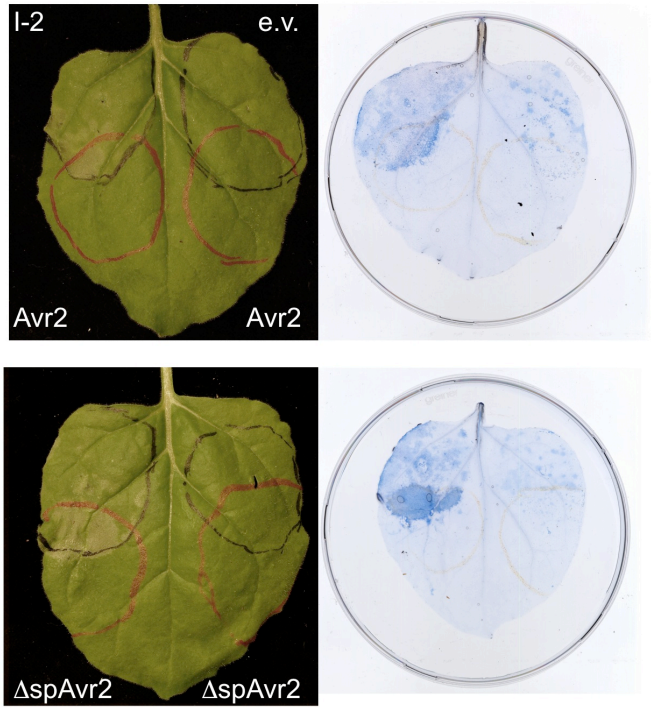

Figure 4. In the presence of I-2, Avr2 induces an HR, with or without its signal peptide for secretion. *N. benthamiana* leaves were agro-infiltrated with constructs for full length Avr2 (Avr2) or Avr2 lacking the signal peptide for secretion (ΔspAvr2). The upper circles indicate the regions infiltrated with *A. tumefaciens* carrying either a binary construct containing *I-2* (left side of the leaf) or an empty vector (e.v. - right side of the leaf). The lower circles indicate the AVR2 or ΔspAVR2 infiltrated zones. The genes are co-expressed at the overlap. The photographs on the left were taken 3 days after agro-infiltration. The photographs on the right show representative leaves from the same experiment taken 2 days after agro-infiltration and stained with trypan blue to visualize cell death.

the xylem vessels, and appears not to be associated with the hypersensitive response (HR) that is commonly observed after activation of *R* genes (Beckman and Mueller, 1987; Mes et al., 2000). Nevertheless, expression of mutationally autoactivated *I-2* can induce HR in leaves of *Nicotiana benthamiana* after agroinfiltration (de la Fuente van Bentem *et al.*, 2005). We therefore reasoned that, in this heterologous system, activation of I-2 by Avr2 may also trigger HR. This turned out to be the case: when expressing *I-2* and *AVR2* in partially overlapping areas in *N. benthamiana* leaves, HR was observed only in the overlap between the two areas where both genes are co-expressed (Figure 4, top panel). As no HR was

observed in the region expressing either *I-2* or *AVR2*, this result indicates that expression of both genes is required for the response, confirming that they for a gene-for-gene pair.

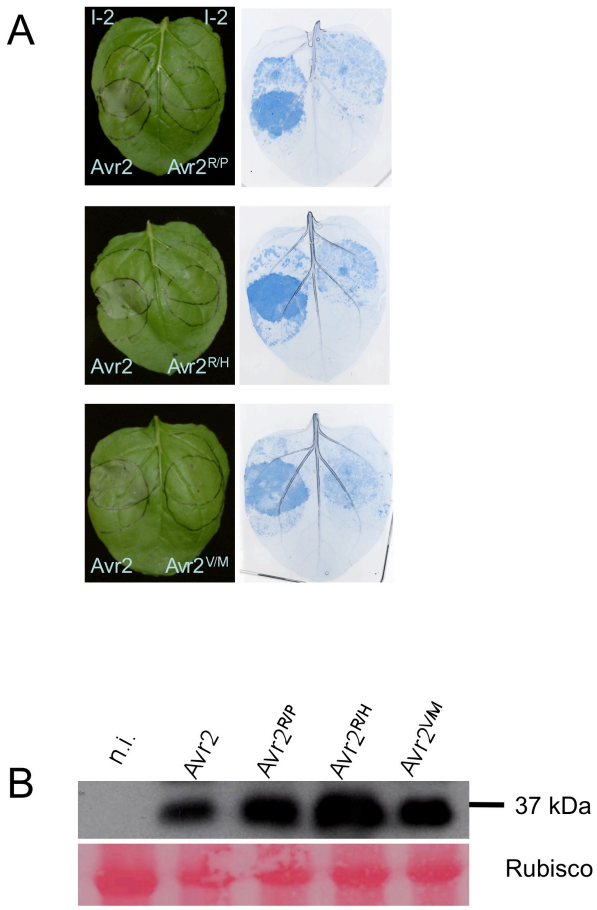

Figure 5. ***AVR2* variants from race 3 *Fol* strains do not trigger HR upon co-expression with *I-2*. A)** *N. benthamiana* leaves agro-infiltrated with constructs for I-2 (upper circles) and ΔspAvr2 (lower circles). The left side of each leaf is agro-infiltrated with a construct that produces wild-type (HR-inducing) Avr2 and the right side with a construct that produces the indicated race 3 variant. Leaves were photographed two days after infiltration (left panels) and cell death was visualized by trypan blue staining of the same leaves (right panels). **B)** Western blot showing the presence of TAP-tagged ΔspAvr2 in agro-infiltrated *N. benthamiana* leaves. One day after agro-infiltration total protein extracts were subjected to SDS-PAGE, followed by immunoblotting and detection with a PAP antibody recognizing the TAP tag. The first lane contains proteins isolated from non-infiltrated (n.i.) leaves. The remaining lanes contain proteins from leaves expressing the indicated *AVR2* construct. Ponceau S staining of Rubisco (lower panel) is shown as a measure of the amount of protein loaded in each lane.

Initially, the complete *AVR2* coding sequence was used, which encodes a protein with a signal peptide for translocation into the endoplasmatic reticulum. Avr2 was therefore expected to be secreted by the transformed plant cells into the apoplastic space. This situation resembles normal infection, as Avr2 (Six3) is also present extracellularly, as it was found in the xylem sap (Houterman et al., 2007). I-2, on the other hand, is predicted to be

localized in the cytosol (Simons et al., 1998). To test whether Avr2 can activate I-2 inside

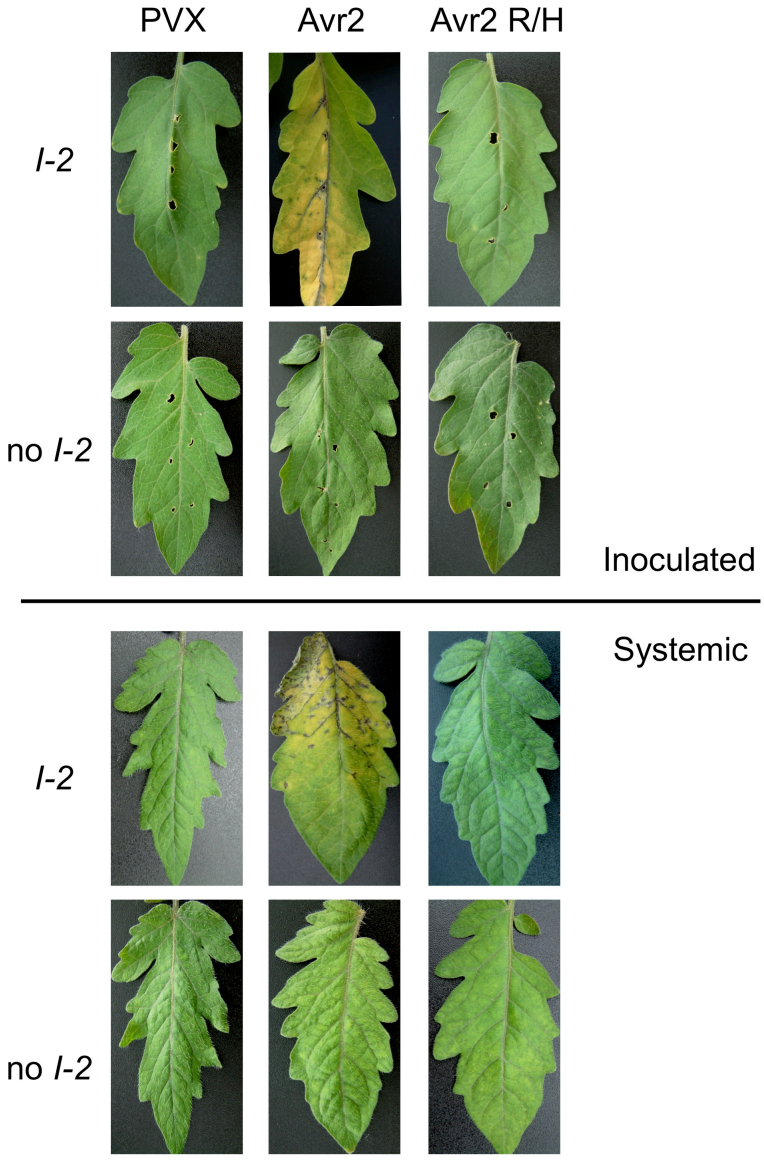

Figure 6. Avr2 causes an HR in tomato only in the presence of *I-2*. Leaflets of tomato were toothpick-inoculated close to main vein with *A. tumefaciens* harbouring T-DNA with PVX. PVX is expressed and spreads systemically throughout the plant. The PVX constructs were either empty ('PVX') or encode either Avr2 (middle) or the virulent Avr2 variant $Avr2^{R/H}$ (both without signal peptide). HR occurs in inoculated as well as systemic leaves only in *I-2* containing plants inoculated with *PVX::AVR2*. The photographs were taken 9 days after inoculation.

plant cells, we agro-infiltrated *N. benthamiana* with a construct encoding a truncated Avr2 form lacking the signal peptide for secretion. This truncated version also triggers I-2-dependent HR (Figure 4, bottom panel). It even does so slightly faster than the secreted version does, which explains the slightly more intense HR visible in Figure 4 with the truncated version. Based on these data, we conclude that Avr2 can be recognized by I-2

inside the plant cell.

Next, we co-expressed *I-2* with the *AVR2* variants found in race 3 strains, using *AVR2* constructs without a signal peptide. As shown in Figure 5a, none of the three *AVR2* mutants triggered HR when co-expressed with *I-2*. To exclude the possibility that the absence of a response is due to weak expression or instability of the variants, we also used TAP (tandem affinity purification)-tagged versions. Fusion of wild-type *AVR2* to the TAP-tag coding sequence did not affect its ability to induce *I-2*-dependent HR (results not shown). Western blot analysis of total protein extracts isolated from the agroinfiltrated leaves shows that Avr2 mutant proteins are accumulating in the leaves, apparently to the same level or even in somewhat higher amounts as wild-type Avr2 (Figure 5b). These experiments demonstrate that each of the three single amino acid changes found in Avr2 of various race 3 strains prevents activation of I-2 even though the proteins are stably produced.

To investigate the effect of recognition of Avr2 by I-2 in the natural host (tomato), we expressed *AVR2* systemically in tomato using a Potato virus X (PVX)-based expression system. As we knew that Avr2 without signal peptide triggers HR effectively in *N. benthamiana* (Figure 4), PVX::ΔSP-*AVR2* was introduced into tomato leaves by toothpick inoculation with *Agrobacterium tumefaciens* carrying a binary vector containing the recombinant PVX construct (Takken *et al.*, 2000). Expression of the 'intracellular' *AVR2* version (i.e. not encoding a signal peptide for secrection) was found to trigger HR in both inoculated and systemic leaves. This HR was specific for *I-2*-containing plants, as it did not occur in plants without the resistance gene. Also no HR was observed using the race 3 using the $AVR2^{R/H}$ allele (Figure 6). We conclude that tomato responds to activation of endogenous I-2 in leaves with cell-death response.

Discussion

Identification of Avr2, together with the cloning of *I-2* ten years ago (Simons *et al.*, 1998), completes the first *AVR*/*R* gene pair of a xylem-colonizing fungus. All three avirulence factors matching the major introgressed *Fol* resistance genes in tomato (*I*, *I-2* and *I-3*) have now been identified. Avr1, Avr2 and Avr3 are, together with other small proteins of yet unknown function, secreted into xylem sap during host colonization (Houterman et al., 2007). In race 3 strains, which overcome *I-2*-mediated disease resistance, *AVR2* carries point mutations at one of three positions. These mutations do not affect virulence of *Fol* but prevent recognition of Avr2 by I-2, thereby uncoupling virulence and avirulence.

The occurrence of three independent point mutations indicate that *I-2*-breaking race 3 strains have emerged at least three times over the last decades (Cai *et al.*, 2003). In principle, race 3 could arise through loss of *AVR1* from a race 1 isolate that already had a mutated

(non-I-2 activating) version of *AVR2* (*AVR1 avr2* → *avr1 avr2*). However, we did not find polymorphisms between *AVR2* genes of diverse race 1 and race 2 strains. Apparently, the virulence of (many) race 1 strains towards an *I-2*-contaiing tomato is caused by suppression of *I-2* function by *AVR1* rather than mutation of *AVR2* (Houterman *et al.*, 2008). It is therefore likely that race 3 has repeatedly evolved from race 2, requiring only a singe mutation in *AVR2* to become virulent towards an *I* / *I-2* containing cultivar (Figure 7). The commonly used combination of *I* and *I-2* is therefore predicted to be durable only when the *Fol* population in an area is of race 1. A combination of *I* and *I-3* may be more durable because *AVR3* appears to be less prone to mutations that prevent recognition by *I-3* (Rep *et al.*, 2005).

The three amino acid residues affected by *I-2* evading mutations in Avr2 are in close proximity of each other near the N-terminus of the predicted mature protein (Figure 2). This region is therefore likely to be involved in the recognition by I-2. A direct interaction between Avr2 and I-2 is a possibility, but so far we have not been able to obtain evidence for this using yeast two-hybrid or pull-down experiments (results not shown). As strains carrying mutations in *AVR2* are fully virulent, it is likely that the I-2-evading Avr2 versions still interact with a host 'target' protein and interfere with its function. In line with the guard model (Van der Biezen and Jones, 1998), I-2 might recognize the complex of Avr2 and this target protein (a virulence target), and the mutations in Avr2 may alter the I-2 interaction surface, preventing recognition of this complex. An alternative explanation is that the mutations in Avr2 interfere with interaction with an I-2-guarded homolog of its presumed virulence target (recently termed a 'decoy'; van der Hoorn and Kamoun, 2008)) without affecting its interaction with the virulence target itself. Future experiments aimed at the identification of Avr2-interacting plant proteins will hopefully resolve its recognition mechanism.

As an Avr2 version lacking the signal peptide can activate I-2 to trigger HR, it is likely that Avr2 is taken up by plant cells from the xylem sap during colonization of tomato by *Fol*. These xylem contact cells would then initiate a defense response. Interestingly, in contrast to leaf cells, the defence response of xylem contact cells upon Avr recognition does not usually result in an HR but mainly involves callose deposition, accumulation of phenolic compounds and formation of tyloses and gels in vessels (Beckman and Mueller, 1987; Mes *et al.*, 2000). The latter responses could correspond to the browning of the major vessels and yellowing of the leaves, possibly due to dysfunction of the clogged vessels, as observed her upon PNX::ΔSP-*AVR2* inoculation of an I-2 tomato plant (Figure 6).

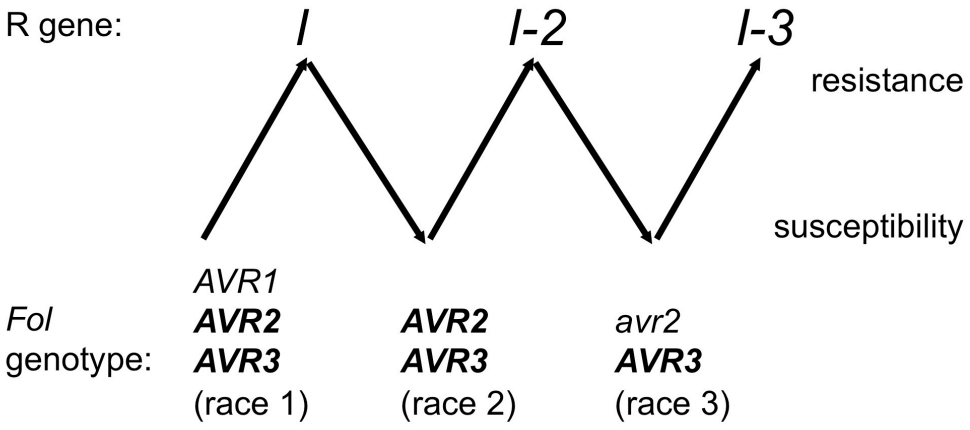

Figure 7. Evolution of *Fol* races in agriculture. This model for the evolution of races of *Fol* in agriculture is based on the presence and sequence of *AVR1*, *AVR2* and *AVR3* genes in a worldwide collection of Fol strains (see text). Introduction of the *I* gene led to resistance against *Fol*, but also to selection of strains that had lost *AVR1* (i.e. race 2). Introduction of *I-2* conferred resistance to race 2, which was overcome by single point mutations in *AVR2* (shown as '*avr2*'), leading to emergence of race 3. In race 1 strains, no polymorphisms were found in *AVR2*, suggesting that race 3 did not emerge directly from race 1 strains by loss of *AVR1*. Note that *I-3* not only confers resistance to race 3 but also race 2, and that neither *I-2* nor *I-3* confer (full) resistance to race 1, because *AVR1* suppresses *I-2* and *I-3*-mediated resistance.

Uptake of a fungal protein by plant cells has been shown previously. ToxA, a small protein and host-selective toxin secreted by *Pyrenophora tritici-repentis*, was shown to be imported as a GFP fusion protein into toxin-sensitive wheat mesophyl cells (Manning and Ciuffetti, 2005). AvrL567, a small protein secreted by haustoria of flax rust (*Melampsora lini*) is recognized by NBS-LRR proteins inside plant cells, possibly through a direct interaction (Dodds et al., 2004; Dodds et al., 2006). Oomycete effectors carrying an RXLR-DEER motif are also taken up by plant cells, and some of them can be recognized by NBS-LRR surveillance proteins (Kamoun, 2006). The mechanisms mediating import of small proteins by plant cells are still unknown, and their discovery is eagerly anticipated (Ellis et al., 2006).

Material and methods

Plant lines, fungal strains and disease assays

The *Fol* wild-type strains used are described in Table 1. The Fol007*avr2* irradiation mutant is described in (Mes *et al.*, 1999b). The tomato lines used are: C32 (no *I* gene) (Kroon and Elgersma, 1993) and 90E341F (*I-2*) (Stall and Walter, 1965). To assess the ability of *Fol* strains to cause disease in specific tomato lines, either seedling assays or 'older plant assays' were used. In the seedling assay, 10-day-old seedlings of tomato were inoculated with a fungal spore suspension and disease was scored after three weeks as described previously (Rep *et al.*, 2004). The outcome of the

disease assays was quantified in two ways: (1) mean plant weight above the cotyledons, and (2) phenotype scoring according to a disease index ranging from 0 (no disease) to 4 (heavily diseased or dead) (Rep *et al.*, 2004). In the older plant assay, 5-week-old plants were inoculated, after removing part of the root system, with a *Fol* spore suspension (5×10^6 spores ml^{-1}), potted and photographed after 14 days.

Deletion of AVR2 in Fol and complementation of ΔAVR2 mutants with AVR2

To create an *AVR2* gene deletion construct, 717 bp downstream of the *AVR2* open reading frame (directly after the stop codon) was amplified by PCR using primers with *Xba*I and *Bss*HII linkers (5'-AAATCTAGATTCTGTGGCAGTTCCCCTT-3' and 5'-AAAGCGCGCGGTGTGTTGAACAGGTGCT-3') and inserted into the binary vector pRW2h next to the hygromycin resistance cassette (Houterman *et al.*, 2008). Then, 1266 bp of upstream sequence (directly adjacent to the start codon) was amplified using primers with *Pac*I and *Kpn*I linkers (5'-AAATTAATTAATAGGATAACAAAGGGCTAAGA-3' and 5'-AAAGGTACCAGTGGTAAATGTTTAGGCAAG-3'), and inserted on the other side of the hygromycin resistance cassette. The resulting *AVR2* gene deletion construct was transformed to *Fol* using *Agrobacterium* as described earlier (Takken *et al.*, 2004). Colonies that were resistance to hygromycin (Duchefa, http://www.duchefa.com) were selected, and homologous recombination were performed at the *AVR2* locus, resulting in the replacement of the *AVR2* open reading frame with the hygromycin resistance cassette, was checked by PCR using primers inside the hygromycin resistance cassette and outside the *AVR2* flanking regions used for construction of the deletion cassette as described above.

To create an *AVR2* complementation construct, a fragment of 1.7 kb was amplified by PCR containing the *AVR2* open reading frame, 945 of upstream sequence and 258 of downstream sequence using primers with *Xba*I and *Pst*I linkers (5'-AAATCTAGACGATGCCTTGACCGAAAGTT-3' and 5'-AAACTGCAGTCCCCACACAGTATTCT-3'). This fragment was cloned into pRW1p (Houterman *et al.*, 2008). Transformants were selected on zeocin (InvitroGen, http://www.invitrogen.com) based on the presence of a phleomycin resistance cassette in pRW1p. The presence of *AVR2* in the transformants was confirmed by PCR.

Construction of Binary Vectors for transformation of N. benthamiana

Full-length *AVR2* was PCR-amplified from *Fol* genomic DNA using primers FP1748 (5'-AAAAAGCAGGCTCTATGCGTTTCCTTCTGCTTATAG-3') and FP1750 (5'-AGAAAGCTGGGTATCAATCCTCTGAGATAGTAAGATAG-3'). Truncated *AVR2* lacking the signal peptide coding sequence was amplified using FP1749 (5'-AAAAAGCAGGCTGGATGCCTGTGGAAGATGCCGATTCATC-3') in combination with FP1750 as reverse primer. Gateway attB flanks were added by adapter PCR using primers FP872 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3') and FP873 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'). The PCR products obtained were recombined via entry clone pDONR207 (Invitrogen, http://www.invitrogen.com) into binary vector CTAPi (Rohila *et al.*, 2004) by the Gateway protocol for cloning attB-PCR products into destination vectors (Invitrogen). As FP1750 covers the endogenous *AVR2* stop codon, the resulting Avr2

proteins are not translationally fused to the TAP tag. Tagged constructs were made using FP1751 (5'-AGAAAGCTGGGTATCCATCCTCTGAGATAGTAAGATAG-3') as reverse primer, which replaces the stop codon. All PCR primers were purchased from MWG (http://www.mwg-biotech.com), and sequences were confirmed by sequence analysis. Construction of binary CTAPi vectors carrying I-2 and I-2^{D495V} has been described previously (van Ooijen *et al.*, 2008).

For creation of the PVX::*AVR2* constructs, *AVR2* lacking the signal peptide coding sequence was PCR-amplified using FP2189 (5'-GTAGGCGCGCCATGCCTGTGGAAGATGCCGATT-3') (*Asc*I site underlined) and FP1750. PCR products were digested with *Asc*I and ligated into the binary PVX vector PSfinx (Takken *et al*, 2000), which was digested with *Asc*I and *Sma*I. All constructs were confirmed by sequencing.

Agrobacterium-mediated transient transformation of *N. benthamiana*

A. tumefaciens strain GV3101(pMP90) (Koncz and Schell, 1986) was transformed with binary constructs as described previously (Takken *et al.*, 2000) and grown to an absorbance at 600 nm of 0.8 in LB-mannitol (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 2.5 g l^{-1} NaCl, 10 g l^{-1} mannitol) supplemented with 20 μM acetosyringone and 10 mM MES pH 5.6. Cells were pelleted by centrifuge at 4000 g at 20 °C for 20 min and then resuspended in infiltration medium (1×MS salts, 10 mM MES pH 5.6, 2% w/v sucrose, 200 μM acetosyringone) and infiltrated at an absorbance at 600 nm of 0.1 (for *I-2* constructs) or 0.5 (for *AVR2* constructs) into 4-5-week-old *N. benthamiana* leaves.

Monitoring of HR on tomato plants carrying a PVX-based expression system

The binary *PVX::AVR2* constructs were transformed to *A. tumefaciens* GV3101 containing the helper plasmid pIC-Sarep (Jones *et al.*, 1992). Transformation of *A. tumefaciens* and its toothpick inoculation onto tomato plants were performed as described by Takken *et al*. (2000). Three-weeks-old tomato plants with *I-2* (line 90E341F) or without *I-2* (C32) were used. Inoculated and systemic leaves were scored from day 7 for the development of necrotic symptoms.

Trypan Blue Staining

Leaves were boiled for 5 min in a 1:1 mixture of ethanol and 0.33 mg ml^{-1} trypan blue in lactophenol, and destained overnight in 2.5 g ml^{-1} chloral hydrate in water.

Protein Extraction and Western Blotting

Infiltrated leaves were harvested and pooled 24 h after agro-infiltration, and frozen in liquid nitrogen. After grinding the tissue, it was allowed to thaw 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 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://calbiochem.com) to obtain a total protein lysate. Samples were mixed with Laemmli sample buffer, and equal amounts of total protein were run at 13% SDS–PAGE gels and blotted on PVDF membranes using semi-dry blotting. Skimmed milk powder (5%) was used as a blocking agent. A 1:8000 dilution of anti-TAP tag antibody (PAP, P1291, Sigma P1291) linked to horseradish peroxidase was used. The signal was visualized by using ECL and BioMax MR film (Kodak, http://kodak.com).

Supplementary data

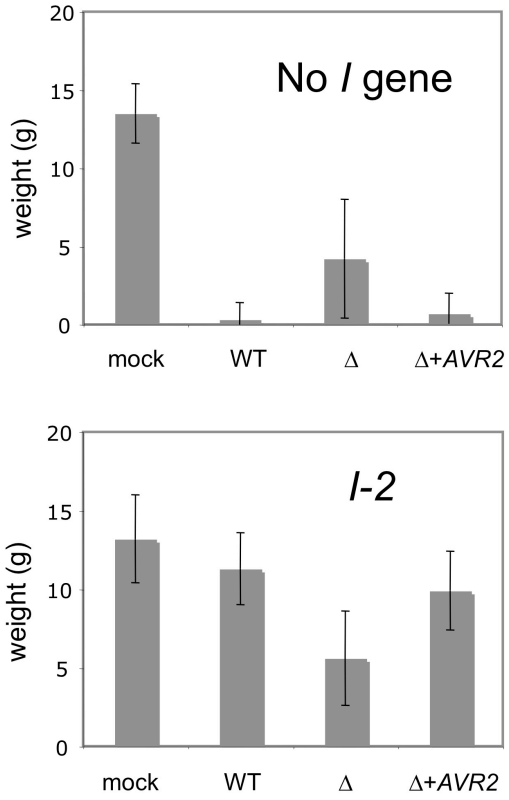

Figure S1. Reintroduction of *AVR2* in an *AVR2* knock-out strain restores virulence and avirulence.
Ten day old seedlings of tomato plant lines without resistance genes to *Fol* (no *I* gene) or with only the *I-2* gene (*I-2*) were inoculated with race 2 isolate Fol007 (WT), a mutant derived from Fol007 in which the *AVR2* gene was deleted (Δ) or an *AVR2* knock-out strain re-transformed with the *AVR2* gene (Δ+*AVR2*). As a control, both plant lines were also mock-inoculated (mock). Average weight above cotyledons of 20 plants was measured for each inoculation three weeks after infection (error bars indicate standard deviation).

Acknowledgement

We thank Lotje van der Does for critical review of the manuscript. Harold Lemereis and Ludek Tikovsky are gratefully acknowledged for plant care. G.v.O. was supported by the Research Council for Earth and Life Sciences, with financial aid from the Netherlands Organization for Scientific Research, and by the Centre for BioSystems Genomics, the Netherlands. F.L.W.T. is supported by financial aid from the Centre for BioSystems Genomics from the Netherlands Organization for Scientific Research, and by the EU Framework 6 program Bioexploit.

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