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Research

The *AVR2–SIX5* gene pair is required to activate *I-2*-mediated immunity in tomato

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Summary

- Plant-invading microbes betray their presence to a plant by exposure of antigenic molecules such as small, secreted proteins called 'effectors'. In *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) we identified a pair of effector gene candidates, *AVR2-SIX5*, whose expression is controlled by a shared promoter.
- The pathogenicity of *AVR2* and *SIX5 Fol* knockouts was assessed on susceptible and resistant tomato (*Solanum lycopersicum*) plants carrying *I-2*. The I-2 NB-LRR protein confers resistance to *Fol* races carrying *AVR2*.
- Like Avr2, Six5 was found to be required for full virulence on susceptible plants. Unexpectedly, each knockout could breach *I-2*-mediated disease resistance. So whereas Avr2 is sufficient to induce *I-2*-mediated cell death, Avr2 and Six5 are both required for resistance. Avr2 and Six5 interact in yeast two-hybrid assays as well as *in planta*. Six5 and Avr2 accumulate in xylem sap of plants infected with the reciprocal knockouts, showing that lack of I-2 activation is not due to a lack of Avr2 accumulation in the *SIX5* mutant.
- The effector repertoire of a pathogen determines its host specificity and its ability to manipulate plant immunity. Our findings challenge an oversimplified interpretation of the gene-for-gene model by showing requirement of two fungal genes for immunity conferred by one resistance gene.

Introduction

Host specificity of the soil-borne plant pathogenic fungus *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) is determined by its lineage specific (LS) pathogenicity chromosomes (Ma *et al.*, 2010; Rep & Kistler, 2010). LS chromosome 14 in *Fol* strain 4287 is required for infection of tomato (*Solanum lycopersicum*) and this chromosome can be transferred during cocultivation from a pathogenic to a nonpathogenic strain, resulting in acquisition of pathogenicity by the recipient strain (Ma *et al.*, 2010; Rep & Kistler, 2010). Analysis of the *Fol* genome revealed that LS chromosome 14 is highly enriched for genes encoding small proteins that are secreted during infection (Ma *et al.*, 2010; Schmidt *et al.*, 2013). Using mass spectrometry, we identified 14 of these 'secreted in xylem' (Six) proteins (Six1-14) in *Fol*-infected tomato plants (Houterman *et al.*, 2007; Schmidt *et al.*, 2013).

Effectors are typically secreted proteins that promote host colonization, often by modulation of plant immunity (Hogenhout *et al.*, 2009; Dodds & Rathjen, 2010). Targeted deletion of either *SIX1* or *SIX3* compromises pathogenicity of the fungus, identifying the encoded Six proteins as genuine effectors (Rep *et al.*, 2004; Houterman *et al.*, 2009). Whereas many effectors suppress immunity, some are recognized by the plant immune system and then trigger defense activation, changing them from virulence into avirulence determinants. For *Fol*, this is the case for Six1, Six3 and Six4; Six1 (Avr3), Six3 (Avr2) and Six4 (Avr1) trigger activation of resistance mediated by *I-3*, *I-2* and *I* or *I-1*, respectively (Takken & Rep, 2010). Notably, Avr1 (Six4) is not required for pathogenicity on tomato plants without resistance genes against *Fol*, but functions as suppressor of *I-2*- and *I-3*-mediated resistance (Houterman *et al.*, 2008). Besides these three Six proteins, Six6 was recently also shown to contribute to pathogenicity of *Fol* and it specifically suppresses I-2-mediated cell death (I2CD) upon transient expression in *Nicotiana benthamiana* (Gawehns *et al.*, 2013). However, no function has been assigned to the other *SIX* genes. Whereas the *SIX* genes are dispersed over LS chromosome 14 and typically have their own promoter region, *AVR2* forms an exception as it shares its 1609 bp upstream region with *SIX5* (Schmidt *et al.*, 2013). In fungi, clustering of genes often implies a common function for the gene products such as biosynthesis or degradation of primary or secondary metabolites (Palmer & Keller, 2010; Slot & Rokas,

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2010). The observed clustering of the *SIX5-AVR2* pair prompted us to investigate whether these two proteins also have a shared function.

Six5 and Avr2 were originally identified in the xylem sap proteome of *Fol*-infected tomato plants. Although Avr2 is secreted during fungal colonization in the water-conducting xylem vessels, the protein can be recognized intracellularly in the plant nucleus by the I-2 protein (Houterman *et al.*, 2009; Ma *et al.*, 2013). I-2 is encoded by the *I-2* resistance gene that is specifically expressed in the parenchyma cells adjacent to the xylem vessels (Mes *et al.*, 2000) and encodes a classical cytosolic NB-LRR protein containing a central nucleotide-binding (NB) domain fused to a C-terminal leucine-rich repeat (LRR) region (Simons *et al.*, 1998). Coexpression of *I-2* and *AVR2* in *N. benthamiana* leaves using agroinfiltration triggers activation of *I-2*, which is visible by a local cell death response (Houterman *et al.*, 2009; Ma *et al.*, 2012). *Fol* strains that break *I-2* resistance carry specific point mutations in the *AVR2* gene (Houterman *et al.*, 2009). Six5, originally identified as 'unidentified protein 2', runs on a two-dimensional gel as a single spot with an apparent mass of 12 kDa (Houterman *et al.*, 2007). After identifying the coding sequence in the genome of *Fol* strain 4287 (the gene was not annotated) the protein was renamed Six5. The mRNA sequence of 360 nucleotides was filed in the NCBI dbase under accession number FJ767863 (Ma *et al.*, 2010). *SIX5*, together with *AVR3* (*SIX1*), *AVR2* (*SIX3*) and *SIX2*, is highly conserved in all known *Fol* isolates (Lievens *et al.*, 2009). We here show that, like Avr2, Six5 contributes to virulence of *Fol* on tomato plants, that Six5 and Avr2 can interact and these two proteins together are required for *I-2*-mediated resistance.

Materials and Methods

Fungal strains, plant materials and bioassay on tomato

For bioassays a susceptible tomato (*Solanum lycopersicum* L.) cultivar (C32) (Kroon & Elgersma, 1993) and a resistant cultivar (90E341F) were used (Stall & Walter, 1965). The *SIX5* knockout was generated in both a Fol007 and a Fol004 background (Houterman *et al.*, 2009). The *AVR2* knockout and complementants have been described before (Houterman *et al.*, 2009). *Fol* inoculations were done according to the root dip method (Mes *et al.*, 1999) and statistical analysis on disease index and plant weight were scored as described before (Houterman *et al.*, 2009).

Generation of the *Fol SIX5* knockout and its complementants

Regions flanking the *SIX5* ORF were amplified from Fol007 genomic DNA using primer pairs FP1505 and FP1506 or FP1507 and FP1508 (Supporting Information Table S1). The upstream region, flanked by *Pac*I and *Kpn*I sites, was cloned in front of the hygromycin cassette present in binary vector pRW2 h (Houterman *et al.*, 2008). The downstream region, flanked by *Xba*I and *Bss*HII sites, was cloned behind the hygromycin gene. The obtained pRW2 hΔ*SIX5* plasmid was used for *Agrobacterium tumefaciens* (EHA105)-mediated *Fol* transformation (Rep *et al.*, 2004). Absence of *SIX5* in hygromycin resistant transformants was confirmed by PCR using primers FP1488/FP1489. In locus insertion of the knockout cassette was confirmed using primer pairs FP745/FP2281 (right border) and FP659/FP2282 (left border).

In order to complement *Fol*Δ*SIX5* the *SIX5* gene including its 1049-bp upstream and 271-bp downstream sequences was PCR-amplified from genomic *Fol* DNA using primers FP1725 and FP1726. The primers added *Xba*I and *Pst*I restriction sites and the obtained product was subcloned into pRW1p. The obtained pRW1pSix5Com was used for agrotransformation and presence of *SIX5* in the zeocin resistant transformants (Houterman *et al.*, 2008) was verified using primers FP1505/FP1957 (promoter region) and FP1488/FP1726 (*SIX5* ORF and terminator).

Vector construction

Binary CTAPi vectors carrying *AVR2* have been described before (Houterman *et al.*, 2009). Δsp*SIX5* was amplified using primers FP2701 and FP2203 from a *Fol*-tomato cDNA library as template (de la Fuente van Bentem *et al.*, 2005). Gateway *attB* linkers were added using primers FP872 and FP873. The Δsp*SIX5* product was recombined into pDONR207 and shuttled into binary vector CTAPi (Rohila *et al.*, 2004) using Gateway cloning (Invitrogen). To generate yeast two-hybrid constructs, the Δsp*AVR2* was amplified from *AVR2*-CTAPi (Houterman *et al.*, 2009) using FP1873 and FP1874. The obtained product, flanked with *Nco*I and *Eco*RI sites, was cloned into the same sites present in pAS2-1 (Clontech, Leusden, the Netherlands). Δsp*SIX5* was amplified from the *Fol*-tomato cDNA library using primers FP3446 and FP3447 and cloned between the *Nco*I and *Eco*RI sites in pACT2 (Clontech). For antigen production the Δsp*AVR3* coding sequence was amplified using FP2297/FP2298. Gateway *attB* linkers were added using primers FP872 and FP873. The amplified fragment was introduced into pDONR207 resulting in pENTR207::Δsp*AVR3*. Entry clones pENTR207::Δsp*AVR2* (Houterman *et al.*, 2009), pENTR207::Δsp*SIX5* and pENTR207::Δsp*AVR3* were used in an LR reaction to shuttle the *Fol* genes to destination vector pGEX-KG-GW (Dhonukshe *et al.*, 2010) using Gateway cloning (Invitrogen). The pGEX-KG::Δsp*AVR2,* pGEX-KG::Δsp*SIX5* and pGEX-KG::Δsp*AVR3* encode proteins carrying an N-terminal GST tag. To enable *SIX* gene expression in *N. benthamiana* pENTR207::Δsp*AVR2* (Houterman *et al.*, 2009) and pENTR207::Δsp*SIX5* were recombined into binary vector pGWB451 (Nakagawa *et al.*, 2007). Δsp*AVR3*, without its prodomain, was amplified from pENTR207::Δsp*AVR3* using primers FP2646 and FP2578. The PCR product was digested with *Xba*I and *Bam*HI and cloned in the same sites of SLD3104 (Tameling & Baulcombe, 2007) as a translational fusion with a HA-SBP tag. To generate the BiFc constructs, pENTR207::Δsp*AVR2* and pENTR207::Δsp*SIX5* were recombined into binary BiFc vectors (Gehl *et al.*, 2009). The resulting constructs, pSCYN::Δsp*AVR2*, pSCYC::Δsp*AVR2*, pVYN::Δsp*SIX5*, pVYC::Δsp*SIX5*, pGWB451::Δsp*AVR2* and SLD3104::Δsp*AVR3*, were transformed to *A. tumefaciens*

GV3101 according to (Ma *et al.*, 2012). All PCR primers were purchased from MWG (http://www.mwg-biotech.com) and all clones were verified by sequencing.

Agroinfiltration of *N. benthamiana* leaves

Agroinfiltration of *N. benthamiana* leaves was performed as previously described (Ma *et al.*, 2012). For immunoblotting, *A. tumefaciens* GV3101 was infiltrated at an OD_{600} of 1.0 and proteins were extracted 36 h after infiltration. For BiFc, *A. tumefaciens* GV3101 containing the *p19* gene of tomato bushy stunt virus was included as a silencing suppressor (Voinnet *et al.*, 2003). Coinfiltration of *A. tumefaciens* strains containing the BiFC vectors and *p19* were carried out at a final OD_{600} of 0.5, 0.5 and 1.0 with 1 : 1 : 1 mix ratio. To visualize cell death, *A. tumefaciens* was infiltrated using an OD_{600} of 0.2 (*I-2* construct) or 0.5 (*AVR2* or *SIX5* construct), respectively. Leaves were scored 3 d post inoculation (dpi) and stained for cell death using trypan blue (Ma *et al.*, 2012).

RNA isolation and reverse transcriptase (RT)-PCR analysis

For RT-PCR analysis water or *Fol* inoculated tomato seedlings were grown on vermiculite (Agra-vermiculite, Rhenen, the Netherlands). At 10 dpi, roots were harvested and pulverised in liquid nitrogen. RNA was isolated using TRIzol LS reagent (Invitrogen) and subsequently purified using RNeasy Mini spin columns (Qiagen). At the same time DNA was removed with RNase-free DNase (Qiagen) using the on-column approach. cDNA was synthesized using the M-MuLV Reverse Transcriptase RNase H minus kit (Fermentas Thermo Scientific, St Leon-Rot, Germany). Primer combinations FP962/FP963 and FP1993/FP1994 were used to amplify *AVR2* and *SIX5*, whereas *FEM1* gene was amplified using primer set FP157/FP158.

Xylem sap collection and SDS-PAGE

For xylem sap collection 4-wk-old C32 tomato plants were inoculated with Fol004, Fol029, Fol007, Fol007Δ*AVR2*, Fol007*ΔAVR3* and Fol007Δ*SIX5* and xylem sap was collected 14 dpi as described (Rep *et al.*, 2002; Krasikov *et al.*, 2011). Briefly, stems were cut below the second true leaf and sap dripping from the cut surface was collected in tubes placed on ice for 6 h. The collected xylem sap was centrifuged to remove contaminating spores and soil and subsequently stored at −20°C.

For label-free protein quantification 25 plants per inoculum were inoculated with water, Fol007, Fol007Δ*AVR2* and Fol007Δ*SIX5*. Xylem sap was isolated as described earlier from four independent biological replicates. A fraction of the sap was used for immunoblotting and the remains were concentrated with a Centricon plus-70 (Millipore) unit to a final volume *c.* 200 µl. The protein concentration was determined with the bicinchoninic acid method (Sigma Aldrich). After trichloroacetic acid/aceton precipitation protein isolated from inoculated plants with water, Fol007, Δ*AVR2* and Δ*SIX5*, respectively, was dissolved in sample buffer at equal concentration (1.5 µg μl^{-1}) and 30 µl per sample was loaded on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed with Hoefer Mighty Small SE250 minigel equipment (Amersham Bioscience). After a short run, Coomassie Page-Blue (Fermentas) was used to visualize the proteins in the SDS-PAGE.

Yeast two-hybrid assays

The matchmaker GAL4 two-hybrid system and yeast strain PJ694a were used for analysing protein interactions. Yeast transformation was performed using lithium-acetate and polyethylene glycol 3350 (Gietz & Woods, 2002). Eight colonies were transferred from MM-WL plates, lacking Trp and Leu, to MM-HWL and MM-AWL plates lacking Trp, Leu, His and Ade. Plates were incubated for 7 d at 30°C after which one colony per combination was used to inoculate 1 ml MM-WL. Following a 36 h incubation the cells were spun down and resuspended in 25 µl 0.9% NaCl to an OD_{600} = 1 and 6 µl was spotted on MM-WL, MM-AWL and MM-HWL plates. After 4 d incubation at 30°C the plates were photographed. Total yeast protein was extracted according to (Horvath & Riezman, 1994).

Bimolecular fluorescence complementation (BiFc)

For the BiFC assay, infiltrated *N. benthamiana* leaves were harvested 36 h after infiltration. The leaf discs were analysed using a LSM510 confocal laser scanning microscope equipped with a × 40/1.2 water-immersion objective (Zeiss, Jena, Germany). Excitation of the fluorophore was done at 458 nm for SCYN/SCYC, 488 nm for SCFP/Venus or 514 nm for the Venus/Venus combination using an argon laser. Emission was captured with a 470–500 nm, 505–530 nm or 520–555 nm pass filter, respectively. Images were scanned eight times.

Generation of polyclonal antibodies

pGEX-KG::Δsp*AVR2*, pGEX-KG::Δsp*SIX5* and pGEX-KG::Δsp*AVR3* were transformed to *E.coli* BL21 (DE3). To induce expression 1 mM IPTG was added when the cells reached an OD_{600} of 0.8. Following a 3 h incubation at 18°C cells were harvested (5000 ***g***, 4°C, 15 min) and suspended in lysis buffer (1 mg ml^{-1} lysozyme, 0.2% triton X-100 in PBS, pH = 7.4) supplemented with protease inhibitor (complete; Roche). The suspension was incubated at 4°C for 20 min with gentle agitation and sonicated 4 × 20 s followed by centrifugation (13 000 ***g***, 4°C for 30 min). The cleared homogenate was incubated with glutathione-sepharose (GE healthcare, Freiburg, Germany) and the recombinant protein was eluted with 20 mM reduced glutathione, 150 mM NaCl and 50 mM Tris-HCl at pH 8.5. One mg of purified Avr2, Avr3 and Six5 protein with GST tag was used for immunization of two rabbits following the high-speed protocol (BioGenes, Berlin, Germany). Only rabbits were used of which their preimmune sera did not cross-react with plant proteins in a range of 10–30 kDa that were isolated from *N. benthaminana*, *S. lycopersicum* and *Arabidopsis thaliana* leaves.

Protein extraction and western blotting

For xylem sap analysis, 20 µl sap was loaded on a 15% TRIS-Tricine gel (Schagger & von Jagow, 1987). After electrophoresis, proteins were blotted onto PVDF membranes using semi-dry blotting. Skimmed milk powder (5%) was used as blocking agent and hybridisation was done in the presence of 0.1% tween-20. Anti-Avr2 antibody was used at a 1 : 10000 dilution, anti-Avr3 at a 1 : 5000 dilution. As secondary antibody Goat-anti-rabbit conjugated with horseradish peroxidase (Pierce, Rockford, IL, USA) was used at a 1 : 5000 dilution. The presence of the antigenic proteins was visualized by ECL using BioMax MR film (Kodak, http://www.kodak.com).

In order to validate the sensitivity and specificity of the polyclonal Six antibodies, total protein was isolated from agroinfiltrated *N. benthamiana* leaves expressing *AVR2-GFP*, *SIX5-GFP* and *AVR3-HASBP* using extraction buffer (9.5 M urea, 100 mM Tris pH 6.8, 2% SDS, 5 mM DTT). Antisera of Avr3 (Six1), Six5 and Avr2 were used at a dilution of 1 : 5000.

In order to assess Six5 and Avr2 accumulation in yeast, 30 µl was loaded on SDS–PAGE and after blotting the membrane was probed either with the Avr2 antibody (1 : 5000 dilution) or horseradish peroxidase-conjugated anti-HA antibody (monoclonal 12CA5; Roche) at a 1 : 3000 dilution.

Mass spectrometry and label-free quantitative proteomics

For each xylem sap sample one gel slice containing all proteins was cut from the coomassie-stained gel. In-gel digestion was performed as described by Rep *et al.* (2002). The peptides obtained after digestion were analysed by nanoLC-MS/MS as described by Lu *et al.* (2011). Raw data from the LTQ-Orbitrap were analysed with MaxQuant software (Cox & Mann, 2008; Hubner *et al.*, 2010) to identify the proteins and allow label-free quantification (LFQ). Default MaxQuant 1.1.36 settings were used according to the description by 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, accessed 3 November 2011) 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 v3 (ftp://ftp.solgenomics.net/../../proteins/protein_predictions_from_unigenes/singlespecies_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). To ensure consistency in sample preparation all protein samples were ran and extracted from one SDS-PAGE gel. In subsequent analysis it was found that this bulk preparation had introduced low amounts of *Fol* derived proteins in the mock-treated samples. Similar low amounts of Six5 and Avr2 were found in the respective knockouts, suggesting cross-contamination during sample preparation. Because the 'contaminant' levels were equal between all samples the data were scaled to an arbitrary set value of 6, at which contaminating *Fol* proteins were no longer visible in the knockout samples.

Results

SIX5 and *AVR2* share an upstream region and Six5 is required for full virulence of *Fol*

SIX5 resides on supercontig 36 of chromosome 14 of the genome of *Fol* strain 4287 (Ma *et al.*, 2010; Rep & Kistler, 2010; Schmidt *et al.*, 2013). The gene is in close proximity of *AVR2* (*SIX3*), sharing an upstream region of 1609 bp (Schmidt *et al.*, 2013). *SIX5* was found to contain three introns (Fig. 1a). In the 1609-bp region between the two genes one *mimp* (miniature impala) transposable element of 222 bp is present 256 bp upstream of *AVR2* and 1131 bp upstream of *SIX5* (Schmidt *et al.*, 2013).

In order to assess the role of Six5 in infection of tomato, a *Fol007SIX5* knockout strain (Δ*SIX5*) was generated using homologous recombination. Screening of over 120 hygromycin resistant transformants resulted in the identification of a single knockout strain (see the Materials and Methods section). This very low recombination frequency is in line with that found before for replacement studies at the *SIX5/AVR2* locus (Houterman *et al.*, 2009; Ma *et al.*, 2013; Schmidt *et al.*, 2013). Next, virulence of the Δ*SIX5* strain was assessed by inoculating susceptible tomato seedlings. Cultivar C32 does not carry any resistance gene against *Fol* and inoculation with the wild-type (WT) race 2 isolate Fol007 resulted in typical Fusarium disease symptoms such as wilting and stunting (Fig. 1b). The Δ*SIX5* strain displayed reduced disease symptoms compared with WT as is also apparent from the higher weights and lower disease index of the infected plants (Fig. 1b, right panel). Reintroduction of *SIX5* into the Δ*SIX5* background (Δ+*SIX5*) restored pathogenicity in three out of four transformants, which confirms that the reduced pathogenicity of Δ*SIX5* is caused by deletion of *SIX5* (Fig. 1b). Taken together, Six5 is required for full pathogenicity of *Fol* on tomato qualifying it as a genuine effector.

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

A single intergenic region that drives transcription in two opposite directions is a feature observed before in eukaryotes – coexpression often indicates a functional relationship between the gene products (Kensche *et al.*, 2008; Yang *et al.*, 2008). Therefore, we investigated whether Six5, like Avr2, is also involved in gene-for-gene resistance against *Fol*. Tomato seedlings that carry the *I-2* resistance gene were inoculated with the Δ*SIX5* strain. To our surprise, the Δ*SIX5* strain was able to cause disease on *I-2* plants (Fig. 1c). Reintroduction 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 susceptible plants (see earlier). This result confirms that loss of avirulence is caused by

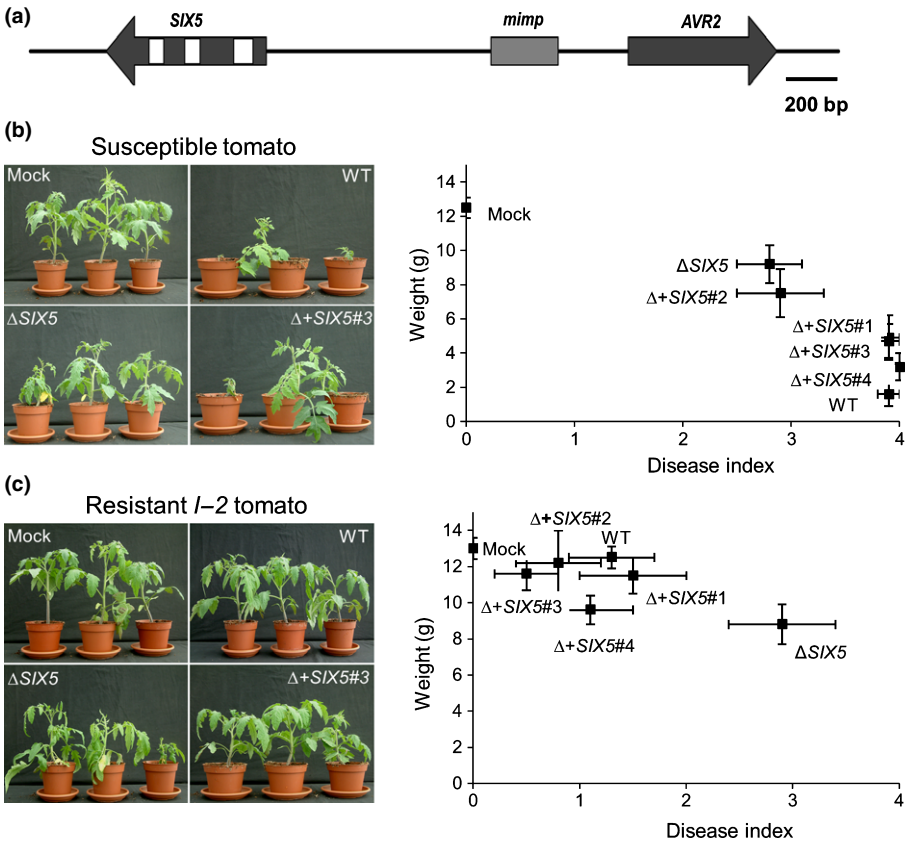

Fig. 1 A *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) *SIX5* knockout is partially impaired in virulence and evades *I-2*-mediated resistance. (a) Schematic representation of the *AVR2-SIX5* locus in *Fol*. *AVR2* and *SIX5* share the same promoter region and are transcribed in opposite directions. White blocks in *SIX5* represent introns; mimp, miniature impala (transposon). (b) Deletion of *SIX5* impairs the fungal pathogenicity, as shown by the reduced disease index and weight of infected tomato (*Solanum lycopersicum*) plants when compared with infection with wild-type (WT) control or complemented knockout isolates. Ten-day-old seedlings of susceptible tomato cultivar C32 were inoculated with mock (water), Fol007 (WT), a Fol007 knockout in which the *SIX5* gene was deleted (Δ*SIX5*) or with complementants in which *SIX5* was reintroduced (Δ+*SIX5*#1-4). Representative plants were photographed 3 wk after inoculation (left panel). Average disease index of 20 plants 3 wk after inoculation was plotted against their mean weight (right panel). (c) Deletion of *SIX5* compromises *I-2*-mediated resistance as depicted by the reduced disease index and plant weight as compared with infection with either the WT or complemented knockout isolates. Ten-day-old seedlings of a resistant tomato cultivar carrying *I-2* were inoculated with the strains indicated. Representative plants were photographed 3 wk after inoculation (left panel) and average disease index of 15 plants 3 wk after inoculation was plotted against their mean weight (right panel). Error bars indicate ± SE.

deletion of *SIX5* (Fig. 1c). Deletion of *SIX5* in race 1 isolate Fol004 did not result in a strain that overcomes *I*-mediated resistance, but did again result in a loss of avirulence towards *I-2* plants (data not shown). We conclude that besides *AVR2* also *SIX5* is specifically required for *I-2*-mediated resistance in tomato, revealing a functional link between the two gene products.

Six5 and Avr2 physically interact with each other

Because Six5 and Avr2 are both required for *I-2*-mediated resistance we set out to examine whether there is a physical interaction between the two proteins and whether such an interaction differs between the virulent and avirulent Avr2 allele. To examine whether Six5 and Avr2 physically interact, the GAL4-based yeast two-hybrid system was used. *AVR2* was expressed as bait and *SIX5* as prey protein. Transformed yeast cells containing the bait and prey plasmids were selected on plates lacking tryptophan and leucine. Expression of the proteins in yeast was confirmed by western blots probed with either the Avr2- or HA-antibody, recognising the HA-tag fused to Six5 (Fig. 2b). Interaction between the two effectors allows the yeast strain to grow on medium lacking histidine or adenine. As shown in Fig. 2(a), neither Six5 nor Avr2 alone were able to complement the histidine or adenine auxotrophy in yeast, but their combination enabled growth on histidine selection, but not on adenine selection, suggesting a weak interaction. Besides Avr2, also a virulent Avr2 allele ($Avr2^{R45H}$), isolated from a *Fol* race 3 isolate that overcomes *I-2*-mediated resistance, interacted with Six5. These results show that the ability of a virulent Avr2 allele to evade I-2 recognition is not correlated with an inability to interact with Six5.

In order to assess the interaction between these two proteins *in planta*, bimolecular fluorescence complementation was employed. Arabidopsis Cnx6, a protein able to form homodimers was used as a positive control for fluoresce complementation. N- or C- terminally tagged VYN::*Cnx6* and SCYC::*Cnx6* were coexpressed in *N. benthamiana* (Gehl *et al.*, 2009). As shown in Fig. 3(a), a green fluorescent signal was observed in the nucleus

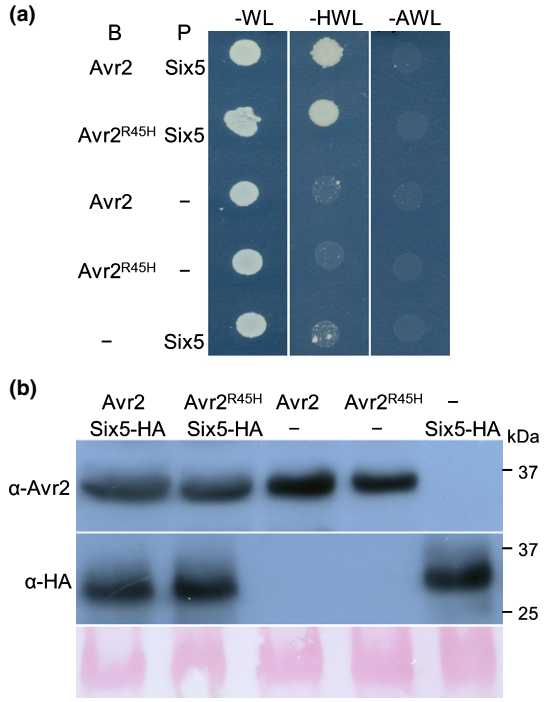

Fig. 2 Six5 and Avr2 interact in the yeast two-hybrid system. (a) Yeast pPJ694a cotransformed with bait (B) or prey (P) vectors that are either empty (-) or carry *AVR2*, an *I-2* breaking variant of *AVR2* (*AVR2*^{R45H}) or *SIX5*. Whereas all double transformants grow on -WL plates, only those coexpressing *SIX5* and either *AVR2* grow on -HWL plates, indicative of an interaction between both proteins. (b) Immunoblotting and detection with Avr2 or HA antibody confirmed the presence of Avr2, Avr2^{R45H} (upper panel) and Six5-HA (middle panel) in the respective strains. Ponceau S staining (lower panel) shows equal protein loading.

and cytosol of the Cnx6 control, but not when SCYC::*Cnx6* was coexpressed with VYN::Δsp*SIX5* (Fig. 3b). These results indicate that fluorescence is specific and due to homodimerization of Cnx6 and is not caused by unspecific interactions of the YFP fragments.

Previously, we reported that Avr2 is able to form homodimers in yeast and *in planta* (Ma *et al.*, 2013). This propensity allowed testing whether the SCYFP-tag does not interfere with the ability of Avr2 to form a protein complex. In cells coexpressing SCYN::Δsp*AVR2* and SCYC::Δsp*AVR2* strong blue fluorescence was observed (Fig. 3c), confirming the formation of Avr2 homodimers in this system. The fluorescence was observed in both the cytoplasm and the cell nucleus, which corresponds with the previously reported subcellular localisation of GFP-tagged Avr2 in *N. benthamiana* (Ma *et al.*, 2013) The complementation shows that the SCYFP tag fused to Avr2 protein does not interfere with its ability to dimerise, making Avr2 itself a suitable positive control for the assay. To test whether Six5 can also dimerise VYN::Δsp*SIX5* and VYC::Δsp*SIX5* were coexpressed, but this pair was unable to complement fluorescence, thereby serving as a negative control for the BiFC assay (Fig. 3f). Next, it was assessed whether Avr2 and Six5 can interact with each other, and although the fluorescent signal was much weaker of that of the Avr2 dimer, green fluorescence was observed for both the SCYN::Δsp*AVR2*/VYC::Δsp*SIX5* and the VYN::Δsp*SIX5*/SCYC::Δsp*AVR2* combination (Fig. 3d,e). The fluorescence pattern largely overlapped with that of the Avr2 multimers in that both cytoplasmic and (weak) nuclear signals were observed. In addition, fluorescent spots were observed at the cell periphery. The identity of these spots is unknown, but time-lapse images show that they are immobile excluding cell organelles as a target for the Avr2-Six5 complex (data not shown). Together these data strongly suggest that Avr2 and Six5 can form a complex *in planta*, in line with the interaction between these two proteins observed in yeast.

Avr2 accumulates in xylem sap of *Fol* Δ*SIX5* -inoculated tomato plants

A possible explanation for the requirement of *SIX5* in *I-2*-mediated resistance is that Six5 modulates expression of *AVR2*. To test whether *SIX5* is required for expression of *AVR2*, or *vice versa*, expression of both effector genes was analysed using RT-PCR in the reciprocal *Fol* knockouts and complemented strains (Houterman *et al.*, 2009; Schmidt *et al.*, 2013). Because *AVR2* is expressed predominantly in root- and xylem-colonizing hyphae (Ma *et al.*, 2013) RNA was extracted from inoculated tomato roots. As shown in Fig. 4(a), expression of *SIX5* in the Δ*AVR2* strain was comparable to that in the WT *Fol*. Likewise, deletion of *SIX5* did not affect expression of *AVR2*, demonstrating that the ability of Δ*SIX5* to break *I-2* is not simply attributable to a lack of *AVR2* expression.

The observed functional overlap between Avr2 and Six5 and their ability to interact *in planta* 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 analyse whether Avr2 accumulation in the xylem was affected in the Δ*SIX5* knockout we isolated xylem sap from infected tomato 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 anti-Avr3(Six1) 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-Avr3 or GFP-tagged Six5 or Avr2 proteins in extracts of agroinfiltrated *N. benthamiana* leaves transiently expressing the corresponding gene (Fig. S1).

The generated antibodies were then used to detect the presence of the three Six proteins in xylem sap of the *Fol*-infected tomato plants described earlier. Immunoblotting showed a strong signal for the Avr3 protein in xylem sap of wild-type *Fol*-infected plants and a weaker signal for Δ*AVR2*- and Δ*SIX5*-infected plants (Fig. 4b, top panel). The lower Avr3 level in the two knockouts likely reflects a reduced fungal biomass, in line with the reduced virulence of the knockout strains (Fig. 1b; 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

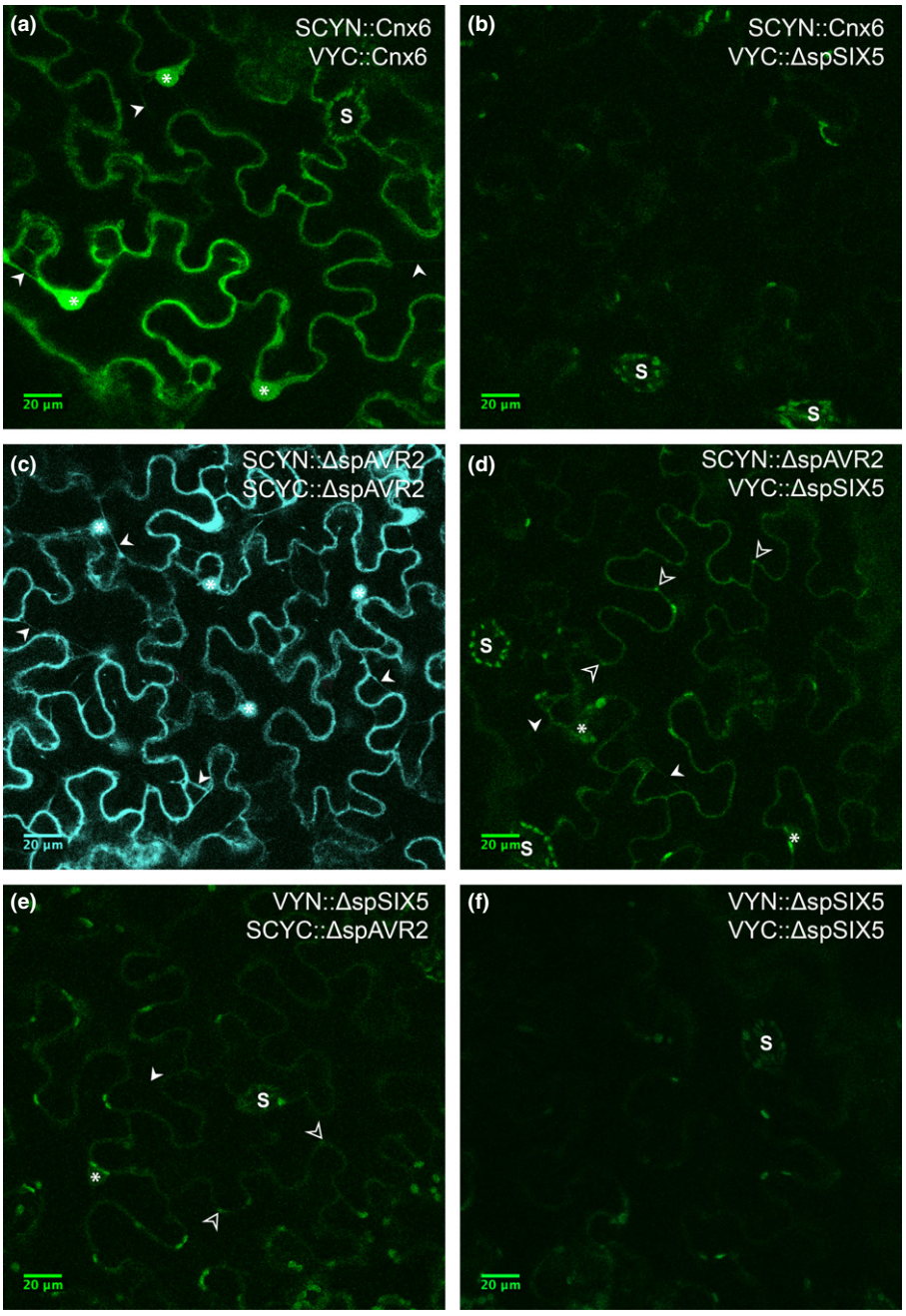

Fig. 3 Avr2 and Six5 interact *in planta*. BiFc constructs of SCYC::Δsp*AVR2*, SCYN::Δsp*AVR2*, VYC::Δsp*SIX5*, VYN::Δsp*SIX5*, SCYN::*Cnx6* and VYC::*Cnx6* were coexpressed in *Nicotiana benthamiana* using agroinfiltration. Fluorescence was detected by confocal microscopy; cyan and green fluorescence represents protein–protein interactions due to complementation of the yellow fluorescent protein (YFP) halves. (a) Green fluorescence following coexpression of the positive controls SCYN::*Cnx6* and VYC::*Cnx6*. (b) SCYN::*Cnx6* and VYC::Δsp*SIX5* do not complement and serve as a negative control. (c) Cyan fluorescence following coexpression of SCYN::Δsp*AVR2* and SCYC::Δsp*AVR2* confirms dimerization of Avr2 *in planta*. (d) Coexpression of SCYN::Δsp*AVR2*/VYC::Δsp*SIX5* or (e) VYN::Δsp*SIX5*/SCYC::Δsp*AVR2* resulted in green fluorescence showing an interaction between Avr2 and Six5. (f) No fluorescence was observed after coexpression of VYC::Δsp*SIX5* with VYN::Δsp*SIX5* suggesting that Six5 does not homodimerise. Green (SCY/VY) and blue (SCY/SCY) fluorescent signals were observed in nuclei (*) and in the cytoplasm as shown by the fluorescent cytoplasmic strands (closed arrows). In the Six5/Avr2 combination spots of unknown identity can be seen at the cell periphery (open arrows). Chloroplasts, abundantly present in the stomata (s), show autofluorescence in the green channel.

control. An identical immunoblot 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. No signal was detected in xylem sap of the Δ*SIX5* strain-inoculated plants, which demonstrates that the Six5 antibody specifically recognizes this effector. Notably, also no band was detected in xylem sap of the Δ*AVR2*-infected plants (Fig. 4b, lower panel), but, in one experiment, upon prolonged exposure, a very faint Six5 signal could be visualized in Δ*AVR2* knockout (Fig. S2), indicating that Six5 does accumulate in the Δ*AVR2* knockout, but apparently at levels around/below the detection limit of our Six5 antibodies. Based on these data, it can be concluded that accumulation of Avr2 in xylem sap does not require the presence of Six5, but it does not exclude the possibility that Avr2 is required to stabilize Six5.

To more precisely quantify the accumulation of the Avr2 and Six5 effector proteins in xylem sap label-free quantitative proteomics was employed. This method allows quantification of Six5 and Avr2 by comparing their abundance to the other fungal and plant proteins detected in the xylem sap. Four-week-old tomato plants were inoculated with water, Fol007, Δ*AVR2* and Δ*SIX5* strains. Two weeks after inoculation, the xylem sap of 25 plants per treatments was collected. Approx. 45 µg of protein was recovered from concentrated xylem sap, which was subjected to nanoLC-MS/MS analysis proteomic measurements and MaxQuant analysis. Four independent biological replicates were

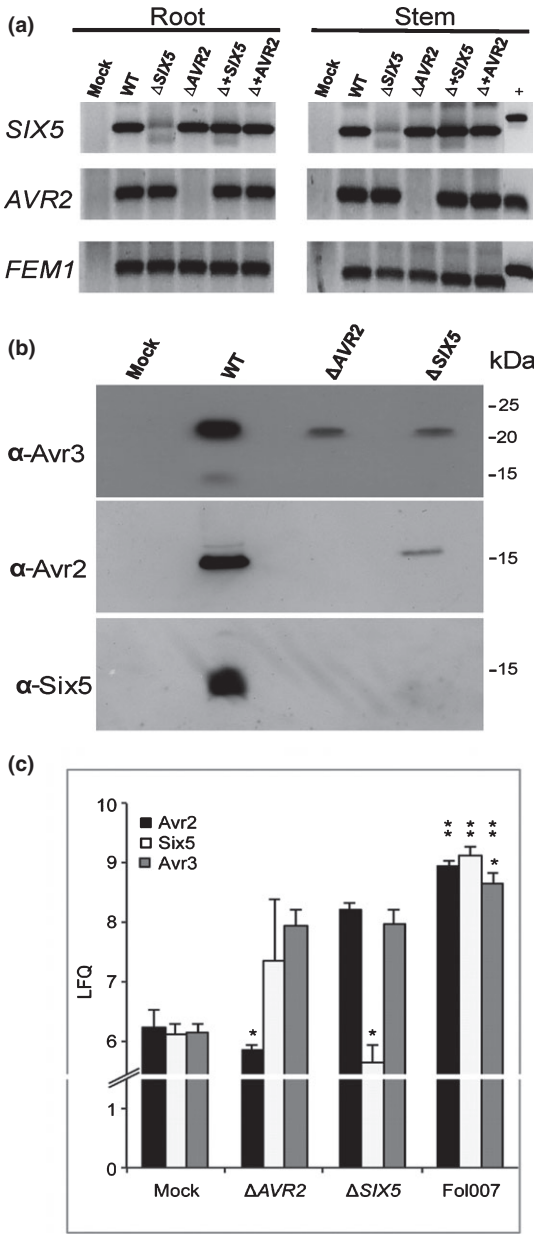

Fig. 4 *AVR2* and *SIX5* are expressed and accumulate in the xylem sap of respective Δ*SIX5* and Δ*AVR2*-infected C32 tomato (*Solanum lycopersicum*) plants. (a) Reverse transcriptase (RT)-PCR analysis of *AVR2* and *SIX5* expression in roots and stems of tomato seedlings infected with either *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) Fol007 wild-type (WT), the *SIX5* and *AVR2* knockouts (Δ) and the respective complementants (Δ+). gDNA of *Fol* (+) and its *FEM1* gene are used as controls. Both effector genes are expressed in the reciprocal knockouts. (b) Western blot of xylem sap isolated from tomato plants C32 inoculated with mock (water), Fol007 WT, Δ*SIX5* or Δ*AVR2 Fol* probed with respectively the Avr3, Avr2 and Six5 antibody. Six5 accumulation is not detectable in the *AVR2* knockout (but could occasionally be detected after overexposure; Supporting Information Fig. S2), whereas Avr2 accumulation is unaffected in the *SIX5* knockout. (c) Label-free quantification (LFQ) of Six5, Avr2 and Avr3 amounts in the xylem sap of 4-wk-old tomato plants inoculated with water (Mock), Fol007 (WT), Δ*AVR2* and Δ*SIX5 Fol*, respectively. Normalized LFQ amounts of Six5, Avr2 and Avr3 are plotted and show that Six5, Avr2 and Avr3 are present in high amounts in the Fol007-inoculated plant, although accumulation of Avr3 is significantly lower than that of Six5 and Avr2. In the mock controls trace amounts of Avr2 and Six5 were observed at levels similar to that in the respective knockouts due to minor cross contamination during sample preparation. Accumulation of Six5 and Avr2 in the reciprocal knockouts remained unaffected as compared with that of Avr1, which serves as a proxy for the amount of fungal biomass. Error bars represent + SD of four independent replicates; except for Δ*AVR2* where three replicates were used. Significant difference in accumulation of Six5, Avr2 and Avr3 within a single treatment: *, $P < 0.05$; significant difference of Six5, Avr2 and Avr3 between different treatments: **, $P < 0.05$.

performed and the obtained spectra were matched to the SGN tomato protein database and a *Fusarium* protein database. LFQ of the identified proteins was performed and values were log2 transformed. A principle component analysis (PCA) revealed a clear separation between the mock-treatment, Fol007 and Δ*SIX5* and the Δ*AVR2* knockouts in which PC1 could explain 51.3% of the data. Except for one Δ*AVR2* replicate sample, which was defined as an outlier based on the PCA analysis and excluded from further analysis, both knockouts behaved identical on the PC1 and PC2 axis, validating the dataset.

To quantify accumulation of Six5 and Avr2 in all inoculated samples, the LFQ values for Six5, Avr2 and Avr3 in the mock, Fol007, Δ*AVR2* and Δ*SIX5* were compared in a one-tailed, homoscedastic student *t*-test and plotted in a bar chart (Fig. 4c). Differences were called significant when $P < 0.05$. As expected, accumulation of Six5, Avr2 and Avr3 was significantly higher with Fol007 than with the knockouts. This is in line with the western blotting results (Fig. 4b) and corresponds with the reduced amount of fungal biomass in the knockouts due to their compromised virulence. Notably, Six5 and Avr2 were found to accumulate only in the Δ*AVR2* and Δ*SIX5* knockouts, respectively, whereas Avr3 accumulated in both strains to similar levels. There was no significant difference between the abundance of Six5 and Avr3 in the sap of Δ*AVR2*-inoculated plants, nor was there a difference between Avr2 and Avr3 accumulation in the sap of Δ*SIX5*-inoculated plants. Taken together, deletion of *AVR2* does not seem to significantly affect Six5 accumulation, nor does deletion of *SIX5* affect Avr2 abundance, suggesting that the effectors do not chaperone and stabilize each other, nor require each other for secretion into the xylem sap of the host.

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

As mentioned before, *Fol* race 3 isolates overcome *I-2*-mediated resistance by carrying variant Avr2 alleles that avoid I-2 activation. Currently, three independent *I-2* breaking Avr2 alleles have been reported: V41M, R45H and R46P (Houterman *et al.*, 2009). Whereas deletion of *AVR2* reduces virulence, race 3 isolates are fully pathogenic. Correspondingly, the variant Avr2 alleles are unaffected in their virulence function for *Fol* (Houterman *et al.*, 2009). Because Six5 is required for *I-2*-mediated resistance, we tested whether accumulation of Six5 in xylem sap is affected in race 3 isolates. Xylem sap was isolated from

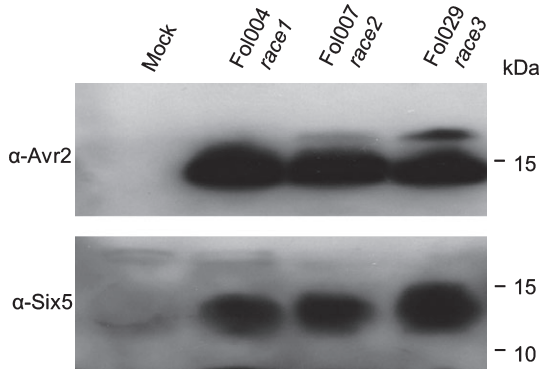

Fig. 5 Avr2 and Six5 accumulate in xylem sap of tomato inoculated with three representative *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) races. Xylem sap was isolated from tomato (*Solanum lycopersicum*) plants inoculated with Fol004 (race 1), Fol007 (race 2) and the *I-2* breaking strain Fol029 (race 3). Xylem sap was subjected to Tris Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotted and probed with an Avr2 or Six5 antibody. The mock-labelled 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.

noninoculated, Fol004 (a race 1 isolate), Fol007 (race 2) or Fol029 (a race 3 isolate carrying the $AVR2^{R45H}$ variant) inoculated tomato plants. Immunoblotting with the Avr2 antibody revealed that, in xylem sap of plants infected with all three races, Avr2 accumulates to similar levels (Fig. 5, top panel). This observation confirms our earlier observation that the Avr2 variants are stable and their ability to escape I-2-mediated recognition is not due to reduced amounts of the protein in the xylem sap (Houterman *et al.*, 2009). Six5 was also found to accumulate in comparable amounts in the three *Fol* races (Fig. 5, lower panel) showing that breaking of *I-2*-mediated resistance by race 3 isolates is not due to reduced accumulation of Six5. These findings confirm that Six5 is required, but not sufficient, for *I-2*-mediated resistance and that induction of resistance requires both an avirulent Avr2 allele and Six5.

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

Activation of an R protein induces host defences that are often accompanied by a local cell death response (Jones & Dangl, 2006; Dodds & Rathjen, 2010). Although cell death is not observed upon plant infection with an avirulent *Fol* strain, a cell death response can be triggered upon heterologous expression of *AVR2* and *I-2* in *N. benthamiana* leaves (Houterman *et al.*, 2009; Ma *et al.*, 2012). To examine whether Six5 can also trigger an *I-2*-dependent cell death response, *SIX5* and *I-2* were coexpressed in *N. benthamiana* leaves using agroinfiltration. Because I-2 is an intracellular protein and Avr2 is recognized inside the plant cell nucleus (Houterman *et al.*, 2009; Ma *et al.*, 2013), an intracellular Six5 variant was used, that is without its signal peptide (Δsp). Fig. 6(a) shows that coexpression of Δsp*SIX5* and *I-2* did not trigger cell death, whereas coexpression of Δsp*AVR2* and *I-2* did.

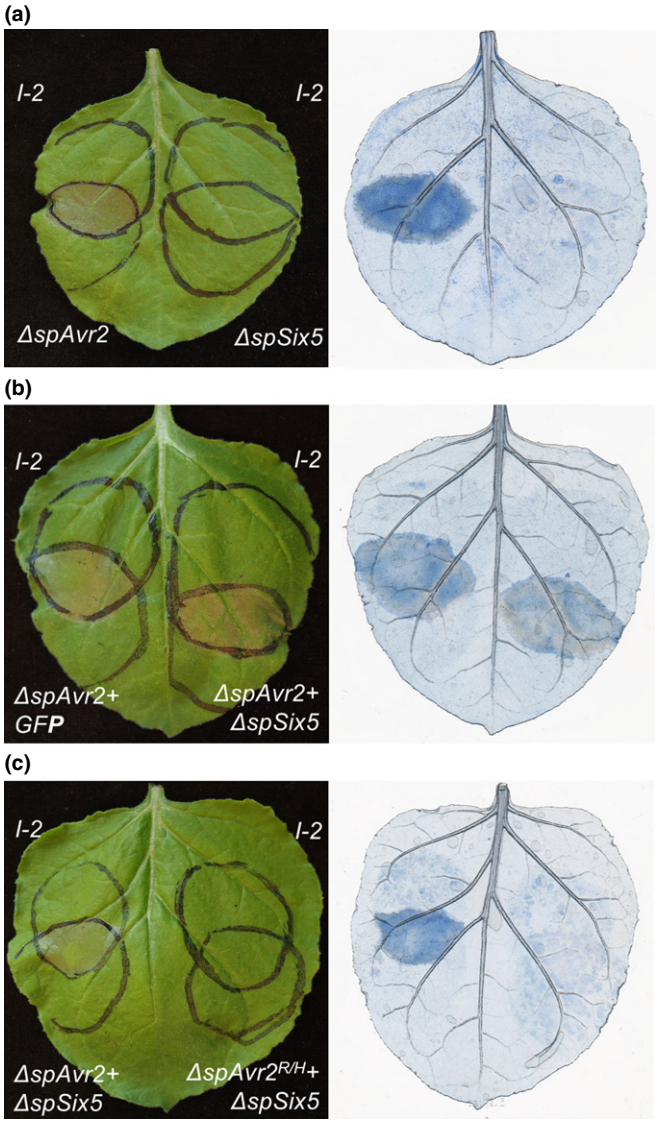

Fig. 6 Avr2, but not Six5, triggers *I-2*-mediated cell death in *Nicotiana benthamiana* leaves when coexpressed via agroinfiltration. (a) Coexpression of *I-2* and *AVR2* lacking its signal peptide for secretion (ΔspAvr2), triggers cell death (necrotic sector) that can be visualized using a trypan blue staining. Coexpression of *I-2* and Δsp*SIX5* does not induce cell death. (b) Coexpression of Δsp*SIX5* with Δsp*AVR2* and *I-2* does not lead to enhanced cell death as compared with the green fluorescent protein (GFP) control. (c) ΔspSix5 does not induce cell death when coexpressed with the Δsp$Avr2^{R45H}$ variants and I-2, demonstrating that Six5 cannot trigger I-2-mediated cell death.

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 (Fig. S3). Also full length (i.e. secreted) Six5 did not trigger I-2-mediated cell death (data not shown).

Coexpression of all three genes, *I-2*, Δsp*AVR2* and Δsp*SIX5*, induced cell death indistinguishable to that of *I-2* and Δsp*AVR2*, demonstrating that Six5 does not alter the dynamics of *I-2*-mediated cell death in this system (Fig. 6b). We also coexpressed Δsp*SIX5* with the Δsp$AVR2^{R45H}$ variant to test whether this gene pair could induce *I-2*-mediated cell death but they could not

(Fig. 6c). We therefore conclude that, whereas Avr2 alone is sufficient to induce I-2-mediated cell death in *N. benthamiana* leaves, the Six5–Avr2 pair is required for I-2-mediated Fol resistance in tomato.

Discussion

Upon infection *Fol* secretes more than a dozen small proteins into the xylem sap of its host (Schmidt *et al.*, 2013). A role in virulence has been demonstrated for four of these (Takken & Rep, 2010; Gawehns *et al.*, 2013). Here we show that also Six5 is required for full virulence as deletion of *SIX5* reduces pathogenicity, which can be restored upon complementation, identifying it as a genuine effector gene.

Together with *AVR2*, *SIX5* forms a gene pair required for avirulence on *I-2* tomato plants. The *SIX5–AVR2* gene pair appears to be unique for forma specialis *lycopersici* as both genes are actually used as markers for *Fol* identification (Lievens *et al.*, 2009). *SIX5* and *AVR2* are located at the same location on chromosome 14, flanked by inverted repeats of unknown function and sharing their upstream region (Houterman *et al.*, 2007; Schmidt *et al.*, 2013). Expression of both effector 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 & Rep, 2009). Furthermore, deletion studies of the shared upstream region of *AVR2* and *SIX5* suggest that *AVR2* and *SIX5* are under control of the same bidirectional promoter (Schmidt *et al.*, 2013). In yeast and humans, gene pairs under the control of a bidirectional promoter are prone to be related by function (Liu *et al.*, 2011) and a functional relation was found to also apply to *SIX5-AVR2* as both are required for avirulence on *I-2* tomato plants. Besides being coexpressed, an interaction between the gene products was found in yeast assays and *in planta*. Attempts to co-immunoprecipitate (Co-IP) this complex from xylem-sap of *Fol*-infected tomato plants or from protein extracts from *AVR2-SIX5* expressing *N. benthamiana* leaves were unsuccessful (data not shown). Possibly, the *in planta* interaction is transient or too weak to survive the purification and washing steps. A weak interaction is also suggested by the relatively weak interaction found in yeast, as growth was observed only on -histidine plates and not on the more selective -adenine plates, as well as the relatively weak fluorescence in the BiFC assay as compared with that of the Avr2 dimer.

The observation that Six5 and Avr2 interact, and are both required for *I-2*-mediated resistance in tomato, are consistent with a model in which I-2 recognizes a Six5–Avr2 complex or with a role for Six5 in chaperoning Avr2 or facilitating its secretion. The second model is, however, disqualified by the proteomics data that show that Avr2 is present in the xylem sap of plants infected with the Δ*SIX5 Fol* strain, which excludes involvement of Six5 in chaperoning or secretion of Avr2 from the fungus into the xylem sap. Alternatively, it is possible that Six5 is required to facilitate interaction between Avr2 and I-2 during infection, for instance by assisting in the delivery of Avr2 into plant cells. We do not favour this hypothesis because Avr2 secreted from plant cells can trigger I-2-mediated cell death in *N. benthamiana* and tomato, implying Six5-independent Avr2 uptake by host cells (Houterman *et al.*, 2009). Accordingly, coexpression of full-length *SIX5* with *AVR2* did not confer enhanced I-2 specific cell death compared with *AVR2* expression alone (data not shown).

The observation that I-2-mediated resistance in tomato requires Six5 and Avr2, whereas Avr2 alone is sufficient for I-2-mediated cell death in tomato and *N. benthamiana,* suggests that these two responses are (partly) uncoupled. In other systems, NB-LRR-mediated cell death and resistance can also be uncoupled, as shown for instance for RPS4 and Mla10 (Heidrich *et al.*, 2011; Bai *et al.*, 2012). In the latter a nuclear localisation of the activated resistance protein is required for resistance whereas a cytoplasmic localisation is sufficient for induction of cell death. For Rx, resistance and HR require Rx to be present in both the cytoplasm and nucleus, suggesting that different signalling events are triggered at these locations that together are required for a full immune response. By analogy, it is possible that Avr2 activates I-2 in the nucleus to trigger cell death (Houterman *et al.*, 2009; Ma *et al.*, 2013) and that a Six5–Avr2 complex activates a cytosolic I-2-mediated response required for disease resistance. Future experiments employing fluorescently labelled proteins could provide insight in the subcellular (co-)localisation of these fungal proteins in host cells during colonisation of the root by the fungus and reveal whether they act as a complex to trigger I-2 resistance or have separate activities in the cell triggering this response.

The requirement of two genes for avirulence in a fungal pathogen constitutes a new variant of the gene-for-gene model. 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 avoiding I-2 activation. We did not find polymorphisms in Six5 in race 3 isolates in our *Fol* collection. Indeed, screening a representative subset of *Fol* isolates from different VCGs that can overcome I-2 mediated resistance and analysing all 17 race 3 isolates in our collection revealed that all carry mutations in the *AVR2* gene (Houterman *et al.*, 2009 and P.M. Houterman *et al.* unpublished data). Possibly, mutations in *SIX5* that allow evasion of recognition also compromise its virulence function. The observation that a gene pair acts together to trigger resistance gene mediated immunity differs from the reverse situation in which one resistance gene mediates recognition of distinct effectors, as for example *Avr1b-1* and *Avr1k* from *Phytophthora sojae* that are both recognized by the soybean *Rps1k*-gene (Song *et al.*, 2013). Another example is the tomato Cf2 resistance protein that guards the Rcr3 protease that is targeted by two distinct effectors: the Avr2 effector from *Cladosporium fulvum* and the Gr-VAP1 protein from the potato cyst nematode *Globodera rostochienis* (Rooney *et al.*, 2005; Lozano-Torres *et al.*, 2012). Likewise, the *A. thaliana* RPM1 protein recognizes two distinct Avr proteins from *Pseudomonas syringae*, AvrB and AvrRpm1, that both target the same RPM1-guarded host protein, RIN4 (Bisgrove *et al.*, 1994; Mackey *et al.*, 2002). In these examples a shared host factor is targeted, but for Avr2 and Six5 it is unlikely that they have a shared host target guarded by I-2, as only Avr2 is able to trigger

I-2-mediated cell death in tomato and *N. benthamiana* (Houterman *et al.*, 2009), whereas Six5 is not. To summarize, although the underlying molecular mechanism on how this protein pair triggers disease resistance remains enigmatic, these findings expand our understanding of the ways in which pathogens may trigger – or avoid – recognition by host immune systems.

Acknowledgements

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Validation of the specificity of Avr3, Avr2 and Six5 polyclonal antibodies using protein extracts of *Nicotiana benthamiana* leaves expressing the corresponding effector gene constructs.

Fig. S2 Six5 accumulates in the xylem sap of *Fol*Δ*AVR2*-infected: tomato plant.

Fig. S3 Six5 accumulates in *Nicotiana benthamiana* leaves after agroinfiltration.

Table S1 Primers used in this study

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