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Comparative genomics of *Fusarium oxysporum* f. sp. *melonis* reveals the secreted protein recognized by the *Fom-2* resistance gene in melon

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Summary

- Development of resistant crops is the most effective way to control plant diseases to safeguard food and feed production. Disease resistance is commonly based on resistance genes, which generally mediate the recognition of small proteins secreted by invading pathogens. These proteins secreted by pathogens are called ‘avirulence’ proteins. Their identification is important for being able to assess the usefulness and durability of resistance genes in agricultural settings.
- We have used genome sequencing of a set of strains of the melon wilt fungus *Fusarium oxysporum* f. sp. *melonis* (Fom), bioinformatics-based genome comparison and genetic transformation of the fungus to identify *AVRFOM2*, the gene that encodes the avirulence protein recognized by the melon *Fom-2* gene.
- Both an unbiased and a candidate gene approach identified a single candidate for the *AVRFOM2* gene. Genetic complementation of *AVRFOM2* in three different race 2 isolates resulted in resistance of *Fom-2*-harbouring melon cultivars. AvrFom2 is a small, secreted protein with two cysteine residues and weak similarity to secreted proteins of other fungi.
- The identification of *AVRFOM2* will not only be helpful to select melon cultivars to avoid melon *Fusarium* wilt, but also to monitor how quickly a *Fom* population can adapt to deployment of *Fom-2*-containing cultivars in the field.

Keywords

virulence gene; *AVRFOM2*; comparative genomics; *Fusarium oxysporum* f. sp. *Melonis*; gene-for-gene interaction; melon *Fom-2* resistance gene

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Introduction

Pathogenic microorganisms constantly evolve strategies to evade the plant's innate immunity that comprises physical barriers, programmed cell death and antimicrobial compound production (Dangl & Jones, 2001). Among these strategies is the employment of small, secreted proteins – often called effectors – to interfere with recognition, suppress plant defence responses or otherwise promote invasion (Lo Presti *et al.*, 2015). Sometimes, effectors act as avirulence proteins. Flor's gene-for-gene model elegantly described the genetic manifestation of this important feature of the plant immune system, which states that for every dominant avirulence (*AVR*) gene in the pathogen, there is a cognate resistance (*R*) gene in the host. The interaction between their gene products leads to the activation of host defence responses, such as the hypersensitive response (HR) that arrests the growth of biotrophic fungi (Flor, 1955).

Melon (*Cucumis melo*) is an important horticultural food crop in subtropical and tropical regions, but is also grown extensively in temperate zone countries. An important disease that results in reduced melon yield and fruit quality is Fusarium wilt disease that is caused by the soil-inhabiting fungus *Fusarium oxysporum* (Oumouloud *et al.*, 2013). This fungus colonizes the roots, followed by extensive growth through the water-conducting xylem vessels, which leads to wilting and ultimately death of the infected plant. *Fusarium oxysporum* has a two-partite genome consisting of a core part that is orthologous to other *Fusarium* species and a highly dynamic lineage-specific part (Ma *et al.*, 2010). The lineage-specific part of a tomato pathogenic isolate, *F. oxysporum* f. sp. *lycopersici* strain 4287, comprises one-fourth of the genome and includes supernumerary chromosomes that can be transferred horizontally (Ma *et al.*, 2010). The lineage-specific chromosomes and chromosomal subregions are enriched in transposable elements and genes involved in host–pathogen interactions (Ma *et al.*, 2010; Schmidt *et al.*, 2013).

Strains of *F. oxysporum* that cause melon wilt are grouped into f. sp. *melonis*. Two resistance genes have been identified in the muskmelon germplasm that confer resistance to a subset of *F. oxysporum* f. sp. *melonis* (*Fom*) strains: *Fom-1* and *Fom-2*. Both *Fom-1* and *Fom-2* were identified by map-based cloning and are used extensively in commercial melon cultivars (Joobeur *et al.*, 2004; Brotman *et al.*, 2013; Oumouloud *et al.*, 2013). *Fom-1* encodes a Toll/Interleukin-1 receptor (TIR)–nucleotide-binding (NB)–leucine rich repeat (LRR) protein (Brotman *et al.*, 2013). *Fom-2* encodes an NB-LRR protein without an *N*-terminal TIR or CC (coiled-coil) domain (Joobeur *et al.*, 2004). Four *Fom* races have been distinguished based on their ability to overcome these resistance genes (Risser *et al.*, 1976). Race 0 can only infect muskmelon cultivars without either *Fom-1* or *Fom-2*. Race 1 is able to infect cultivars harbouring *Fom-1* and race 2 is able to infect *Fom-2*-containing cultivars. Race 1,2 isolates are able to infect cultivars with either resistance gene. For melon growers, it is crucial to know which *Fom* races are present in the soil, in order to plant the appropriate resistant melon cultivars. Based on the gene-for-gene model, these races are expected to be defined by the presence/absence of, or sequence differences in, avirulence genes corresponding to resistance genes *Fom-1* or *Fom-2*.

Some avirulence genes have been identified in the same manner as *R* genes: using sexual crosses and map-based cloning (Luo *et al.*, 2004; Fudal *et al.*, 2007; Gohre & Robatzek, 2008; Parlange *et al.*, 2009). However, for several plant-pathogenic fungi, such as *F. oxysporum*, a sexual cycle has not been observed and this approach is therefore not possible. An alternative approach is to perform a functional screen for fungal proteins that induce a strong immune response culminating in tissue necrosis (De Wit *et al.*, 1985; Wevelsiep *et al.*, 1991; Joosten *et al.*, 1994; Takken *et al.*, 2000). This works when leaf-infiltration of avirulence proteins leads to a hypersensitive response, which for a root-invading pathogen is not necessarily the case. So far, only two studies have resulted in the identification of avirulence genes from root-infecting, asexually reproducing fungi. The first involved the collection of xylem sap from tomato plants that were infected with *F. oxysporum* f. sp. *lycopersici* (*Fol*) and subsequent mass-spectrometry and gene knockout studies to identify the three avirulence proteins that match the tomato *I* (for *Immunity*), *I-2* and *I-3* genes among other effectors (Houterman *et al.*, 2007, 2008). The other successful approach was a comparative genomics approach involving 11 different *Verticillium dahliae* strains of two races that resulted in the identification of the *Ave1* (*Avirulence* on *Ve1*) gene (de Jonge *et al.*, 2012).

Here, we describe the isolation of the *Fom* gene whose product is recognized by the melon Fom-2 resistance protein. We applied comparative genomics including a candidate gene approach based on a miniature transposable element in the promoters of *F. oxysporum* effector genes as well as an unbiased genome comparison approach to identify a single candidate for the *AVRFOM2* gene. Genetic complementation confirmed that this gene indeed engages in a gene-for-gene interaction with *Fom-2*.

Materials and Methods

Plant lines and fungal strains

The following muskmelon (*Cucumis melo* L.) cultivars were used: Cha-T, Cha-*Fom1* and Cha-*Fom2* (a kind gift from ENZA Zaden). The following *Fusarium oxysporum* f. sp. *melonis* W.C. Snyder & H.N. Hansen strains were used (original designation and reference in brackets): Fom001 (NRRL26406; O'Donnell *et al.*, 1998a; Ma *et al.*, 2014), Fom004 (Fom0122; Alvarez *et al.*, 2005), Fom005 (Fom0123; Alvarez *et al.*, 2005), Fom006 (Fom0124; Alvarez *et al.*, 2005), Fom009 (Herman & Perl-Treves, 2007), Fom010 (Herman & Perl-Treves, 2007), Fom011 (Herman & Perl-Treves, 2007), Fom012 (ML2; Perchepied & Pitrat, 2004), Fom013 (Perchepied *et al.*, 2005) and Fom016 (Fom26; Perchepied *et al.*, 2005).

DNA isolation and genome analysis

Fusarium oxysporum genomic DNA was isolated from mycelium that was harvested from 5-d-old NO_3 medium (0.17% yeast nitrogen base, 3% sucrose, 100 mM KNO_3) cultures. Library preparation (170, 500 and 5000 bp inserts) and Illumina sequencing (90 bp paired-end) was performed at the Beijing Genome Institute (BGI, Hong Kong).

Illumina reads were mapped onto the Fom001 genome assembly using CLC Genomics Workbench 'NGS Core Tools – Mapping reads to reference' with default settings except 'similarity fraction = 0.9'. Duplicated reads were removed using PICARD (version 1.93; http://picard.sourceforge.net) and SAMTOOLS SORT (version 0.1.19; http://samtools.sourceforge.net). The breadth and depth of coverage was calculated in 1 kb nonoverlapping windows using BEDTOOLS coverageBed and makewindows (version 2.18.2) (Quinlan & Hall, 2010). Windows were considered absent when the breadth was < 0.2.

The prediction of candidate effectors in the Fom001 genome was carried out as described (Schmidt *et al.*, 2013). Briefly, the genome was searched for a consensus sequence of the Miniature Impala (mimp) inverted repeats (IR). The sequence 2500 bp downstream of the mimp IR was translated in the three possible open reading frames (ORFs). The ORFs bigger than 90 bp were translated and submitted to SIGNALP4.0 (http://www.cbs.dtu.dk/services/SignalP) (Petersen *et al.*, 2011) for signal peptide prediction. If positive, the sequence surrounding the predicted ORF was inspected manually to define the gene model of the candidate effector gene.

RNA isolation and transcriptome sequence analysis

For deep transcriptome sequencing, 10-d-old melon Cha-T seedlings were inoculated with conidia of isolate Fom001 by dipping the roots in the spore suspension for 5 min; roots of infected plants were harvested 10 d after inoculation and flash-frozen in liquid nitrogen. The total RNA of three independent biological replicates was extracted as described previously (Schmidt *et al.*, 2013). cDNA synthesis, library preparation (200 bp inserts) and Illumina sequencing (90 bp paired-end reads) was performed at BGI.

The obtained reads were mapped on the Fom001 genome assembly using CLC workbench 'RNA-seq analysis' with default settings and processed by PICARD (version 1.93; http://picard.sourceforge.net) and SAMTOOLS SORT (version 0.1.19; http://sam-tools.sourceforge.net). Differentially expressed genes were identified using the Bioconductor Package DESEQ (Anders & Huber, 2010).

Data access

All genome and transcriptome raw data have been deposited at the NCBI Sequence Read Archive SRP042982. The *F. oxysporum* Fom001 (NRRL26406) genome sequence is available at The Broad Institute of MIT and Harvard (http://www.broadinstitute.org).

Functional analysis of *AVRFOM2*

T-DNA plasmids to create genetic complementations of *Fom* race 2 isolates were generated by amplifying the *AVRFOM2* ORF and flanking sequences using primers FP5465 (AAATTAATTAAGTATGCATGAGTCTTGTTCACAG) and FP5466 (AAAGGTACCAAGTCAAGCCCTCGAATTG). PCR products were subsequently cloned into pRWh2 (Houterman *et al.*, 2008) between the Pac1 and Kpn1 restriction sites. *Fusarium oxysporum* transformation using Agrobacterium was performed as described (Takken *et al.*, 2004). Subsequently, 10-d-old melon seedlings were inoculated with fungal spore

suspensions as described above and the disease severity was scored 11 d after inoculation by determining the plant fresh weight (FW).

Phylogenetic analysis

The Fom004 genome was assembled *de novo* from the Illumina paired end libraries with 170, 500 and 5000 bp inserts using CLC Genomics Workbench with standard settings. Subsequently, the sequencing reads were aligned to the *de novo* genome assembly using Burrows–Wheeler Aligner (BWA, version 0.7.4-r385 mem) (Li, 2013) and converted to sorted BAM and Mpileup formats using SAMTOOLS (version 0.1.9) (Li *et al.*, 2009). The Genome Analysis Toolkit (GATK, version 2.7-4-g6f46d11) UnifiedGenotyper was used to call variants according to best practices, with reference base calling enabled and the haploid genotyper ploidy setting. A set of 796911 variant sites (criteria: single nucleotide polymorphisms (SNP) in ≥ 1 isolate, and reference or SNP in every isolate) was identified, and a maximum-likelihood tree was constructed from those sites using randomized axelerated maximum likelihood (RAxML, version 7.7.8) (Stamatakis, 2006) using the GTRCAT model with 1000 bootstrap replicates. In addition, we merged all the VCF positions using T_{ABIX} v0.2.6 bgzip, tabix and VCF_{TOOLS} v0.1.9 vcf-merge, to conduct a principal components analysis (PCA) using smartPCA version 9102.

Results

Establishment and characterization of a *Fom* collection

Recently, the genome of one *Fom* isolate (Fom001, NRRL26406, Table 1) has been sequenced and annotated (Ma *et al.*, 2014). To determine the race of this isolate, we performed infection assays on differential melon cultivars. Fom001 is pathogenic on ChaT and Cha-*Fom-1* melon cultivars, but unable to cause disease on Cha-*Fom-2* cultivars and thus belongs to race 1 (Supporting Information Fig. S1). This suggests that Fom001 secretes the putative AvrFom2 protein, which would be recognized by the melon Fom-2 resistance protein.

To identify *AVRFOM2*, we opted for a comparative genomics approach. The first step towards this aim was to collect several *Fom* race 0, race 1 and race 2 isolates. According to the expected gene-for-gene interactions with the melon resistance genes, *AVRFOM2* should be present in race 0 and race 1 isolates and either absent or polymorphic in race 2 isolates. The previously sequenced Fom001 isolate originates from Mexico (O'Donnell *et al.*, 1998a; Lievens *et al.*, 2009). We were kindly provided with three isolates from the south of Spain (Fom004, race 0; Fom005, race 1; Fom006, race 2) (Alvarez *et al.*, 2005), three isolates from Israel (Fom009, race 2; Fom010, race 1; Fom011, race 0) (Herman & Perl-Treves, 2007), another isolate from Spain (Fom013, race 2) (Perchepied *et al.*, 2005) and two isolates for which the origin has not been recorded (Fom012, race 0; Fom016, race 1) (Perchepied & Pitrat, 2004; Perchepied *et al.*, 2005) (Table 1). We confirmed the reported race identities of these isolates by disease assays on differential melon cultivars (Table S1).

Isolates within a *forma specialis* are not always clonally related. The assignment to a certain *forma specialis* is purely based on the ability of an isolate to cause disease on a particular

host plant. Many *formae speciales* are in fact polyphyletic (Kistler *et al.*, 1998; O'Donnell *et al.*, 1998b). To obtain insight into the genetic variation of the isolates available to us, we made nitrate utilization mutants and tested these for their ability to form heterokaryons (complementing the mutant phenotype) with one another, or with a set of tester strains with defined vegetative compatibility groups (VCG). Isolates that belong to the same VCG are part of the same clonal lineage and are able to form heterokaryons (Appel & Gordon, 1996; Glass *et al.*, 2000).

As previously reported, Fom001 belongs to VCG0136 (Lievens *et al.*, 2009). For two isolates from Israel, Fom010 and Fom011, we were not able unambiguously to determine their VCG. The third Israeli isolate, Fom009, belongs to VCG0135. All Spanish isolates as well as Fom012 and Fom016 (unknown origin) belong to VCG0134 (Table 1).

Population structure of the *Fom* collection

Despite the limited genetic and geographic variation of the *Fom* isolates in our collection, we decided to obtain genome sequences. Fom001 was previously sequenced by the Broad Institute (paired-end, 180 bp insert libraries) (Ma *et al.*, 2014); the sequencing reads were downloaded from the sequencing archive (SRX081506) at the NCBI. Paired-end libraries with 170 and 500 bp inserts were sequenced for the other isolates using Illumina HighSeq technology. Additionally, mate-pair libraries with 5 kb inserts were sequenced by Illumina for Fom004 and Fom005 (Table S2). In total, we obtained coverage of the Fom001 genome assembly (54 Mb) ranging from 86× (Fom005) to 139× (Fom004, Table S2).

The sequencing reads were mapped to the genome assembly of Fom001 using parameters that required at least 90% of the read length to be mapped with at least 90% identity. On average, 70–80% of the sequencing reads per isolate could be mapped onto the Fom001 genome assembly, indicating that 20–30% of sequences in the newly sequenced isolates that are absent in the Fom001 assembly (Table S2).

To obtain a detailed view of the sequence variation among the isolates, we determined the SNP sites present in all genomes relative to Fom004 (this genome is more closely related than that of Fom001 to most of the others, allowing more reads to map to the assembly). Based on these 'shared' SNPs, we created a maximum-likelihood tree from those sites using RAxML (version 7.7.8) (Stamatakis, 2006) using the GTRCAT model with 1000 bootstrap replicates (Figs 1, S2, Table S3). As expected, the phylogenetic tree perfectly recapitulates the VCG grouping. All VCG0134 isolates form one clade, while VCG0135 (Fom009) is more related to Fom011 and Fom012. Fom001 (VCG0136) forms a separate clade, with the largest genetic distance to the other isolates (Fig. 1). Apparently, isolates from each country – Spain, Israel and Mexico – form a distinct clade, which might point to independent origins of melon pathogenicity within the *F. oxysporum* species complex at different geographic locations.

Candidate gene approach

In order to identify the *AVRFOM2* gene, we first used a candidate gene approach. In the genome sequence of the reference strain of the tomato pathogen *F. oxysporum* f. sp. *lycopersici* (*Fol*), a miniature transposable element (MITE) resides upstream of all known

effector genes (Schmidt *et al.*, 2013). We demonstrated that this MITE, called mimp, is not required for effector gene expression, but can be used to predict novel effector genes in *F. oxysporum* genomes (Schmidt *et al.*, 2013). We reasoned that a MITE-associated gene might also encode AvrFom2 in the same way as the *Fol* effectors. We therefore searched the Fom001 genome assembly for the inverted repeats of mimp elements. Next, we translated *in silico* 2500 bp downstream of these inverted repeats in the three possible open reading frames (ORFs). These were subsequently searched for signal peptides using SignalP4.0 (Fig. 2a). In total, we identified 11 candidate effector genes encoding small, secreted proteins and harbouring a mimp upstream. Two of these belong to a small gene family (1A and 1B, Table 2).

The predicted products of these 11 candidate effector genes include nine proteins lacking informative annotation or recognizable protein domains, one homologue of the *F. oxysporum* f. sp. *lycopersici* effector Secreted in xylem 6 (Six6) (Houterman *et al.*, 2007) and one protein with a LysM domain (Table 2). Candidates 4 and 5 were not annotated in the Fom001 reference genome, and for candidates 6, 7 and 8 the predicted gene models (FOMG_19741, FOMG_19453 and FOMG_19261) were incorrect. For example, three introns were predicted in FOMG_19621 (candidate 8), but amplification from cDNA indicates the absence of any intron (Fig. S3; Tables S4, S5). The distance between the mimp and the start codon for translation of the candidate effector genes varies between 267 bp (candidate 3) and 1975 bp (candidate 7, Table 2). The size of the encoded proteins ranges from 64 (candidate 4) to 265 (candidate 6) amino acids (corrected gene models, Table 2). *FomLysM* and candidate 8 share the same upstream region including the mimp (i.e. they appear to be divergently transcribed from the same promoter; Table 2).

As is the case for *FomSIX6*, the candidate effectors 1, 4, 5, 6 and 8 have homologues in other *formae speciales* of *F. oxysporum*. Furthermore, candidates 3, 7 and *FomSIX6* have homologues in other fungi, most prominently in *Colletotrichum* species (Table 2). As mentioned above, candidate 1A and 1B belong to a small gene family in *Fom*. A third family member (1C) is present in the Fom001 genome that does not have a mimp upstream of its start codon. We also identified several genes encoding enzymes harbouring a mimp upstream of their start codon, including an astacin-like metalloprotease. This gene is a homologue of *Fol* FOXG_17462, which likewise is associated with an upstream mimp. We predict that a number of the effector candidates identified are involved in the ability of *Fom* to infect melon plants and that one of these could be *AVRFOM2*.

Next, we assessed copy number variation (CNV) and presence/absence polymorphisms for these candidate genes. This was achieved by filtering the whole genome mapping files for each of the isolates to obtain the uniquely mapped and correctly paired reads for the candidate effectors using BedTools (Quinlan & Hall, 2010). We estimated CNV based on the read depth per base. Candidates *1A*, *2*, *FomSIX6* and *FomLysM* are single copy genes in all isolates where they are present (Fig. 2b; Table S4). Candidates *3*, *5* and *7* are either single or multicopy genes in the isolates where they are present (Fig. 2b; Table S4). The read depth per base of multicopy genes is at least twice as high as for single-copy genes of the same isolate. All candidates, except *LysM, 4* and *6* are absent in at least one of the *Fom* isolates (Table S4). Candidates 4 and 6 are present in two or more copies in all *Fom* isolates (Fig.

2b). Except for candidate *6*, the multiple copies of the effector genes are identical. There are three different versions for candidate *6*, involving two SNPs and one single nucleotide deletion.

Genes were considered as absent in an isolate if the sequencing reads covered < 20% of the gene length. Candidate *5* appears to be specific for Fom001 and is absent in all other isolates (Fig. 2b). Candidate *1A* is absent in Fom009 while *FomSIX6* is absent in Fom010 and Fom009. Strikingly, candidate *2* is absent in all race 2 isolates, but present in all race 1 (Fig. 2b) and all race 0 (Table S4) isolates and is therefore a candidate for *AVRFOM2*.

All candidate effector genes are present in the lineage-specific part of the Fom001 genome, i.e. the part that is not covered by sequencing reads of the nonpathogenic Fo47. Candidates *1A* and *FomSIX6* are present in the lineage-specific region that is absent or less conserved in VCG0135 and candidate 5 is present in the Fom001-specific region. All other candidate effector genes reside in contigs that are shared by all *Fom* isolates but are absent in Fo47 (Table S6).

To determine whether the predicted effector genes are expressed during the infection of melon, we isolated RNA from roots of Fom001-infected melon seedlings, sequenced it by Illumina HighSeq and mapped the RNAseq reads to the Fom001 gene models. Except for candidate *5*, all candidate effectors were expressed during melon infection, although *1B*, *6* and *8* were expressed at rather low levels (Fig. 2c). The expression data confirmed that the gene models for FOMG_19741, FOMG_19453 and FOMG_19261 (candidates 6, 7 and 8) were not correct. Based on the read count per gene, candidates *2* and *7* are expressed to a higher level than the other genes. Both from the presence/absence and from the RNAseq analyses, candidate 2 is the prime candidate for the effector protein that is recognized by the *Fom-2* resistance gene.

Unbiased whole genome comparison

To make sure that we had not missed any candidates for *AVR2FOM2* with our candidate gene approach, we also used a whole-genome approach for the identification of *AVRFOM2*. We hypothesized that *AVRFOM2* would be present in all race 0 and all race 1 isolates, because melon cultivars with *Fom-2* are resistant to these races. At the same time, *AVRFOM2* should be absent (or different) in all race 2 isolates, that are not recognized by *Fom-2*-containing melon cultivars. We filtered the genome sequence mapping files of all isolates to extract the correctly paired reads and investigated the Fom004 genome coverage in 1 kb nonoverlapping windows. We considered a window present if sequencing reads covered > 20% of the window length and absent if < 20% of the window length was covered. Next, we grouped the present/absent windows according to races, thereby overruling isolate-specific presence/absence polymorphisms. In total 49600 Fom004-windows were present in all race 0 isolates and 45 401 windows were present in all race 1 isolates. Conversely, 2338 windows were absent in all race 2 isolates compared with Fom004 (race 0) (Fig. 3). To identify candidates for *AVRFom2*, we intersected the windows present in all race 0 and all race 1 isolates with those that were absent in all race 2 isolates. The intersection contained only eleven 1 kb windows. Contigs 36, 519, 1039 and 1259 each contained one window; two windows were present on contigs 55 and 455 and three windows

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on contig 261. There were four ORFs encoded in the 11 windows. Three ORFs did not encode secreted proteins and were therefore not pursued. The previously identified candidate effector 2 was encoded on contig 1259.

Candidate *2* is *AVRFOM2*

Both the candidate gene and the whole-genome approach resulted in the same *AVRFOM2* candidate, coding for a small, secreted protein without recognizable domains that is expressed during melon infection and associated with a mimp (Fig. 4). The candidate gene is weakly similar to *Colletotrichum higginsianum* ChEC13 and *Pyrenophora tritici-repentis* ToxA as discovered by iterative PSI-BLAST searches. Although the overall protein similarity is low, the cysteine residues that form the characteristic cysteine knot in ToxA are conserved (Fig. S4). To confirm that this candidate indeed engages in a gene-for-gene interaction with *Fom-2*, we genetically complemented three different race 2 isolates with the *AVRFOM2* candidate.

First, we cloned the *AVRFOM2* candidate including 1000 bp upstream of the start codon and 1000 bp downstream of the stop codon. Next, we transformed the race 2 isolates Fom006, Fom009 and Fom013 with this construct. Five independent strains that were genetically complemented with the *AVRFOM2* candidate were randomly selected for each of the three isolates. To test whether these race 2 genetic complements were now recognized by *Fom-2*, we inoculated susceptible (Cha-T) and resistant (Cha-Fom2) melon cultivars with a race 1 isolate (Fom001), the three parental race 2 isolates (Fom006, Fom009, Fom013) and the 15 strains genetically complemented with *AVRFOM2*. Eleven days after inoculation we determined the FW of the infected plants. The plant FW of *Fom-2* melon plants infected with all strains that were complemented with *AVRFOM2* closely resembled the weight of the mock-and Fom001 inoculated plants, while all strains were fully pathogenic on susceptible plants (Figs 5a, S5). This experiment confirms that candidate 2 triggers *Fom-2*-based resistance in melon and can hence be designated as *AVRFOM2.*

Discussion

In melon, the resistance protein Fom-2 mediates resistance against race 0 and 1 strains of the vascular wilt fungus *F. oxysporum* f. sp. *melonis*. Identification of avirulence genes has been notoriously difficult in asexually reproducing fungi, because traditional mapping approaches are precluded. Other methods, such as proteomics of infected plant material or leaf infiltration of protein fractions, screening for hypersensitive response and protein purification are laborious and not always applicable. Recently, the *Cladosporium fulvum AVR5* gene was identified by a comparative transcriptomics approach (Mesarich *et al.*, 2014). Here, we employed a population genomics approach to identify a candidate for the avirulence gene corresponding to the melon *Fom-2* resistance gene. Genetic complementation with this gene in the genomes of three different race 2 isolates resulted in recognition by Fom-2 and resistance of the plants to the fungal infection, confirming the identity of this gene as *AVRFOM2*.

For the population genomics approach, we sequenced the genomes of several *Fom* race 0, race 1 and race 2 isolates. Our *Fom* collection did not capture the entire genetic diversity of

Fom worldwide, with nine recognized VCGs (VCG0130–VCG0138) (Jacobson & Gordon, 1988, 1990). Only three or four clonal lines (the VCGs of Fom010 and Fom011 could not be determined) were present in our collection and the collection was dominated by isolates from Spain – all from VCG0134 (Table 1, Fig. 1). Geographic compartmentalization of VCGs has been reported earlier for *Fom* isolates. In Israel, next to VCG0135, isolates associated with VCG0138 are present (Gordon & Martyn, 1997). VCG0134 is the only *Fom* VCG that occurs in multiple countries: Spain, North America and South Africa (our data; Gordon & Martyn, 1997). It has probably been introduced at different sites by human and crop movement (Gordon & Martyn, 1997).

Despite the limited genetic and geographic variation in our set of strains, we were able to identify the same *AVRFOM2* candidate of race 1 by two independent approaches: a candidate gene approach based on previously identified hallmarks of *F. oxysporum* effector genes – a mimp upstream of the start codon and a signal peptide for secretion – and a whole genome comparison by assessing coverage of 1 kb windows of the genome of the race 0 strain Fom004. In contrast to a previous comparative genomic approach by de Jonge *et al.* (2012) our approach did not require *de novo* assembly of the sequenced genomes. We have mapped the genome sequence reads of the different isolates directly onto the newly assembled genome of Fom004 to identify regions present in race 1 and race 0, but absent in race 2 isolates. This limits our approach to regions that are present in this race 0 assembly, but minimizes the effect of assembly errors that can occur because duplicated regions are often collapsed in Illumina assemblies. We obtained only eleven 1 kb windows, thus reducing potential *AVRFOM2* encoding regions tremendously.

The *Fom-2* resistance gene was previously identified by map-based cloning (Joobeur *et al.*, 2004). Using transgenic roots of composite melon plants, the expression of a *Fom-2* promoter fragment was observed along the vascular tissue (R. Perl-Treves, pers. comm.). Similarly, the tomato resistance protein *I-2* is mainly expressed in vascular tissues (Mes *et al.*, 2000). Assuming that *Fom* infects melon plants in a similar manner to *Fol* infecting tomato plants, we can expect that the fungus colonizes the xylem vessels accompanied by secretion of enzymes and effector proteins in the xylem (Houterman *et al.*, 2007). Thus, the expression of *R* genes in vascular tissue appears to be an effective way to recognize effectors and to induce resistance against vascular pathogens.

Pathogens can overcome *R* gene-mediated resistance by different mechanisms. For example, single point mutations in the *AVR2* gene of the tomato pathogen *F. oxysporum* f. *lycopersici* abolish recognition by the corresponding resistance protein I-2 (Houterman *et al.*, 2008). Other effectors are either present or absent in a pathogen population (Stergiopoulos *et al.*, 2007). There are numerous examples of avirulence gene deletions as a means of gaining virulence, reported for fungal plant pathogens such as *C. fulvum* (van Kan *et al.*, 1991; Westerink *et al.*, 2004; Mesarich *et al.*, 2014), *Leptosphaeria maculans* (Gout *et al.*, 2007; Parlange *et al.*, 2009), *Rynchosporium secalis* (Schurch *et al.*, 2004) and *Magnaporthe oryzae* (Pallaghy *et al.*, 1994; Zhou *et al.*, 2007). We did not find any mutation in *AVRFOM2*, instead it is absent in all three race 2 isolates in our collection. It is also absent in four race 1,2 isolates that we have tested (Fig. S6). For *Phaeosphaeria nodorum ToxA*, a host-specific toxin that has an inverse gene-for-gene interaction with its wheat host, both

deletions and point mutations were found to lead to inactivation of the gene in a study involving > 800 isolates (Stukenbrock *et al.*, 2007). We tested only a small number of isolates and cannot exclude that additional race 2 isolates deactivated *AVRFOM2* in another fashion other than gene loss.

Evolution of races in *Fom*

From an evolutionary perspective, it will be interesting to investigate whether race 2 isolates arose independently in different locations, such as Israel and Spain where most of the isolates in our collection came from. In fungi, effector genes are commonly embedded in highly dynamic genome areas. In *Fol*, they reside on lineage-specific chromosomes among numerous transposable elements that appear to facilitate frequent genomic rearrangements (Ma *et al.*, 2010; Schmidt *et al.*, 2013). In many pathogens, overcoming resistance genes in host plants results from insertions, gene deletions and other genetic rearrangements in the vicinity of avirulence genes (Sweigard *et al.*, 1995; Orbach *et al.*, 2000; Gout *et al.*, 2006, 2007; Ridout *et al.*, 2006; Fudal *et al.*, 2007; Khang *et al.*, 2008; Parlange *et al.*, 2009). Finding the structural cause of *AVRFOM2* loss in the different race 2 isolates requires genomes that are assembled to a (near) chromosome level. This represents a challenge especially for the very repeat-rich lineage-specific chromosomes of *F. oxysporum* (Ma *et al.*, 2010). Due to the high number of transposable elements in lineage-specific regions of *F. oxysporum* genomes, the contigs of short-read-based assemblies belonging to these regions are very short. Homologous recombination of transposable elements might be one mechanism leading to the deletion of avirulence genes. In *M. oryzae*, three copies of the *AVR-Pia* gene have likely been deleted by homologous recombination between two *Occan* DNA transposable elements (Sone *et al.*, 2013). Long sequence reads produced by new sequencing technologies such as PacBio or Nanopore sequencing should assemble into longer contigs, providing additional details about genome rearrangements that may have led to the loss of *AVRFOM2*.

The durability of resistance genes is of central importance to sustainable disease management. Deployment of lines with single dominant *R* genes imposes strong selection on the pathogen population structure that can result in adapted pathogen races and subsequent resistance breakdown. Both *Fom-1* and *Fom-2*-containing cultivars are used extensively in commercial muskmelon variants (Oumouloud *et al.*, 2013). Apparently, by loss of *AVRFOM2* the pathogen can overcome resistance of *Fom-2*-containing cultivars. Of course, the primary function of AvrFom2 is probably to contribute to virulence of the pathogen and not its betrayal to the host plant. The AvrFom2 protein has some similarity to effector proteins of other plant pathogenic fungi. Notably, it shares several conserved residues including two cysteine residues with the ToxA protein from the wheat pathogens *Phaeosphaeria nodorum* and *Pyrenophora tritici-repentis* (Friesen *et al.*, 2006) as well as the putative *Colletotrichum higginsianum* effector ChEC13 (Kleemann *et al.*, 2012). *ChEC13* is expressed during penetration of the host cell wall and is thought to be developmentally linked to penetration hypha formation (Kleemann *et al.*, 2012). Perhaps AvrFom2 fulfils a similar role during *Fom* infection of melon.

The dual function of effectors in virulence and avirulence has led to the design of strategies for creating more durable disease resistance in crops. If an avirulence protein fulfils an important virulence function during infection, *R* genes corresponding to these avirulence genes will be more durable, because *AVR* deletion will impose a fitness penalty on the pathogen. Race 2 isolates are not less pathogenic than race 0 or race 1 isolates on melon cultivars without resistance genes, suggesting that loss of *AVRFOM2* imposes only a minimal fitness penalty, if any, for the pathogen. Ultimately, gene knockout of *AVRFOM2* in race 0 or race 1 isolates will be needed to elucidate whether or not AvrFom2 contributes in any way to the severity of wilt disease in melon plants.

Our identification of *AVRFOM2* will not only be helpful for the development of a diagnostic test for race 2 or race 1,2 to provide information about which melon cultivars to employ to avoid disease, but also for monitoring how quickly a *Fom* population can adapt to a *Fom-2* containing cultivar in the field by either jettison or mutation of *AVRFOM2*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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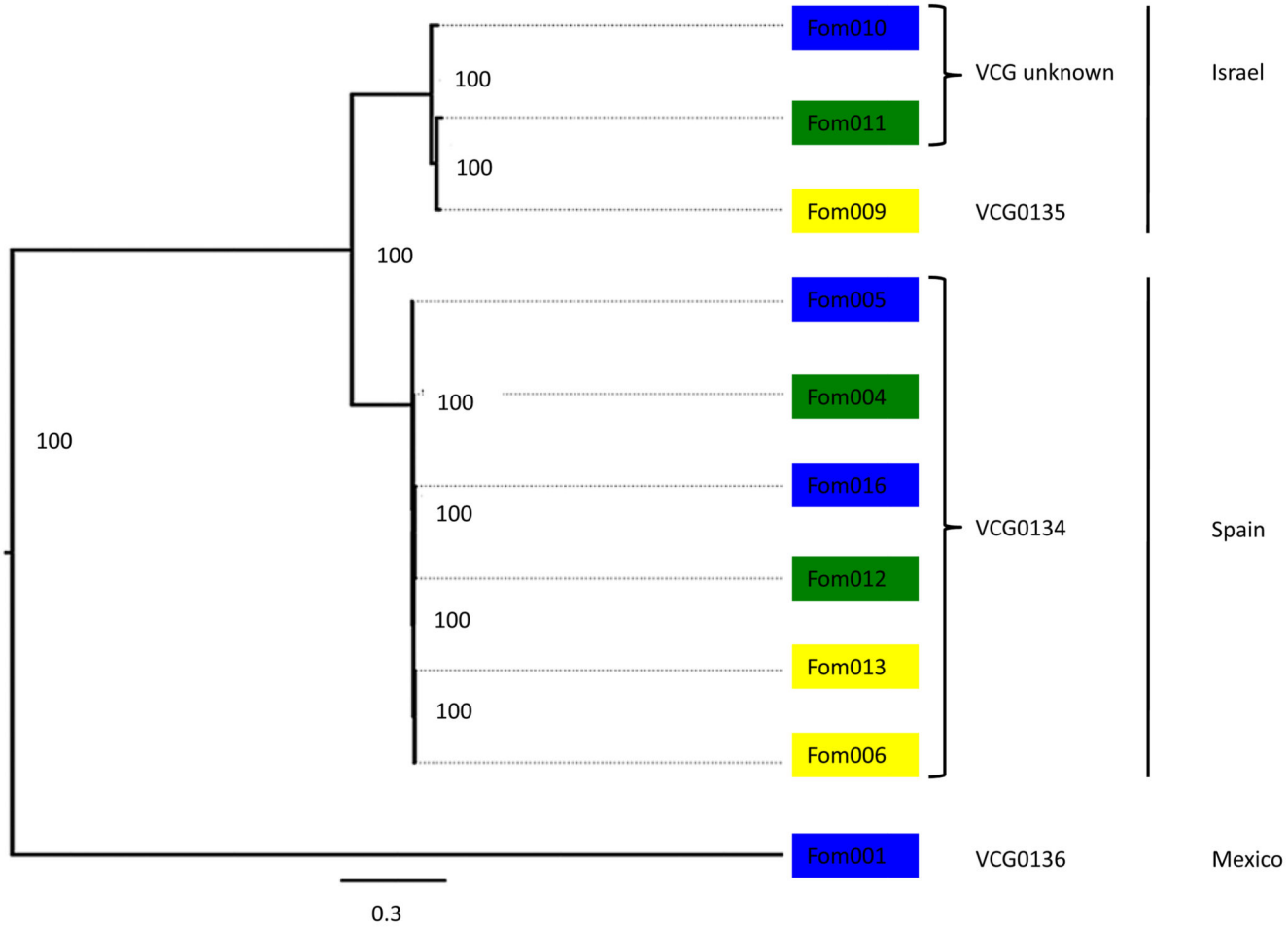

Fig. 1.

Fusarium oxysporum f. sp. *melonis* (*Fom*) isolates cluster according to vegetative compatibility groups (VCGs) and geographic location, but not according to race. The Illumina paired-end sequencing reads were mapped to the Fom004 genome using Burrows-Wheeler Aligner (version 0.7.4-r385 mem) (Li, 2013). Single-nucleotide polymorphisms were called using the Genome Analysis Toolkit (see the Materials and Methods section). The variant positions were used to construct a Randomized Axelerated Maximum Likelihood (version 7.7.8) (Stamatakis, 2006) tree using the GTRCAT model with 1000 bootstrap replicates. Branch length indicates nucleotide substitutions per site. Green boxes, race 0 isolates; blue boxes, race 1 isolates; yellow boxes, race 2 isolates. VCGs and geographic origin of the isolates are shown on the right.

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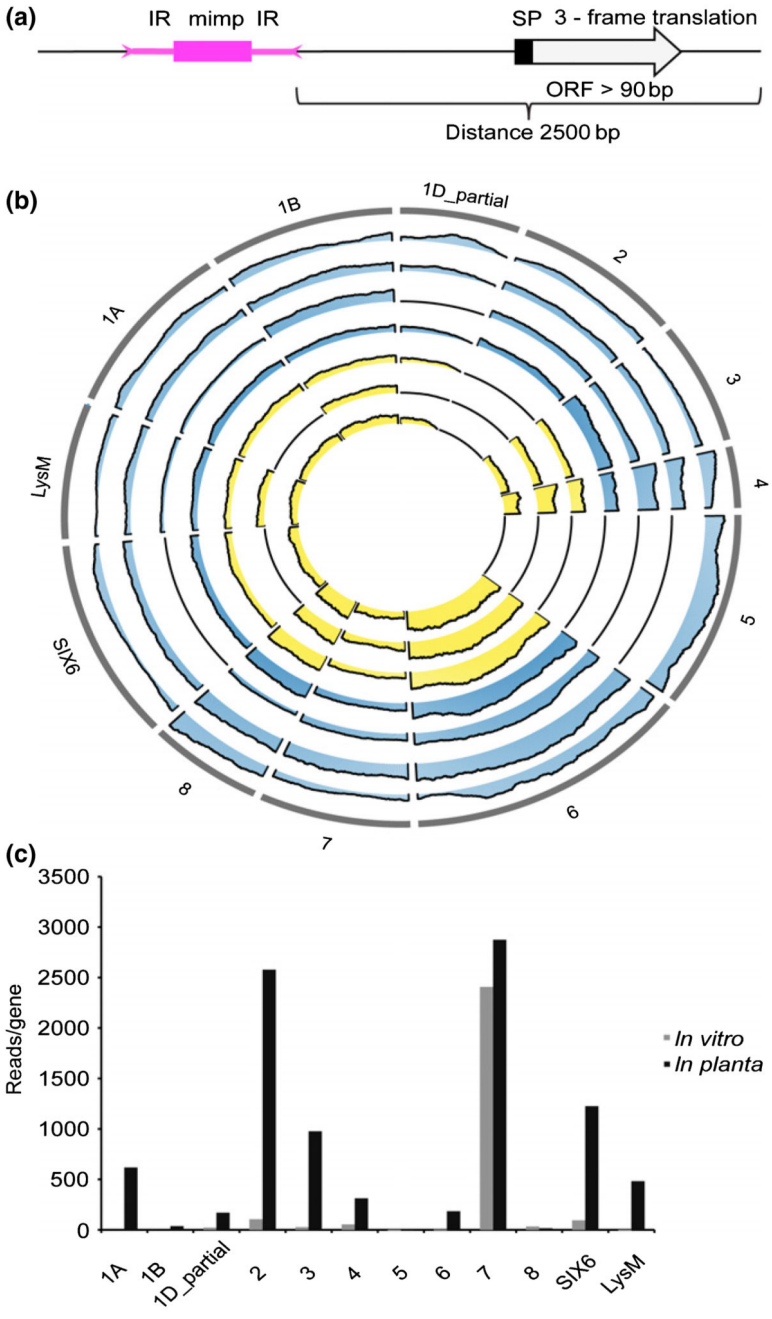

Fig. 2.

The predicted *Fusarium oxysporum* f. sp. *melonis* Fom001 effector 2 is present in all race 1 isolates and absent in all race 2 isolates. (a) Effector genes were predicted by searching the Fom001 genome for inverted repeats (IR) of the Miniature Impala (mimp) transposable elements. The sequence 2500 bp downstream of the mimp IR was translated in the three possible reading frames. Open reading frames (ORFs) > 90 bp were submitted to SignalP4.0 (Petersen *et al.*, 2011) to identify *N*-terminal secretion peptides. (b) The 11 predicted effectors are shown as dark grey lines. The inner histograms show the read depth per base of

the mapped genome sequencing reads per isolate (only correctly paired reads were included). Race 1 isolates are shown in blue, race 2 isolates in yellow (from outside to inside: Fom001, Fom005, Fom010, Fom016, Fom006, Fom009, Fom013). (c) We isolated and sequenced RNA from the roots of Fom001-infected melon seedlings and from *in vitro* grown Fom001. Bar graphs indicate the number of mapped RNA-seq reads for each gene.

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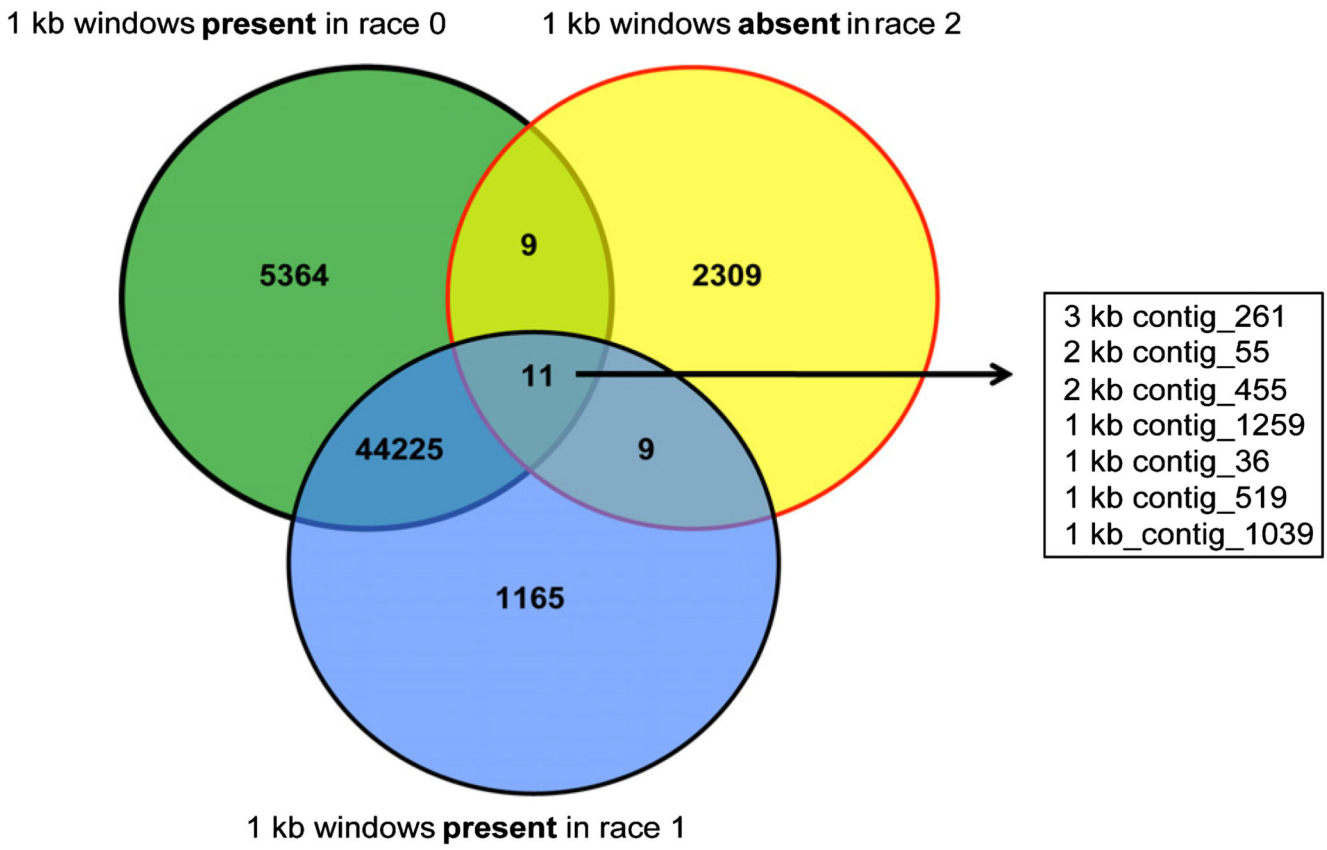

Fig. 3.

Eleven *Fusarium oxysporum* f. sp. *melonis* Fom001 1 kb windows are present in all race 0 and race 1 isolates and absent in all race 2 isolates. Whole genome sequencing reads of all isolates were mapped against 1 kb windows of the Fom001 genome. Windows were considered present when > 20% of the window length was covered by sequencing reads and absent when < 20% of the gene length was covered. Windows either present in all race 0 isolates (green circle), present in all race 1 isolates (blue circle) or absent in all race 2 isolates (yellow circle) were intersected.

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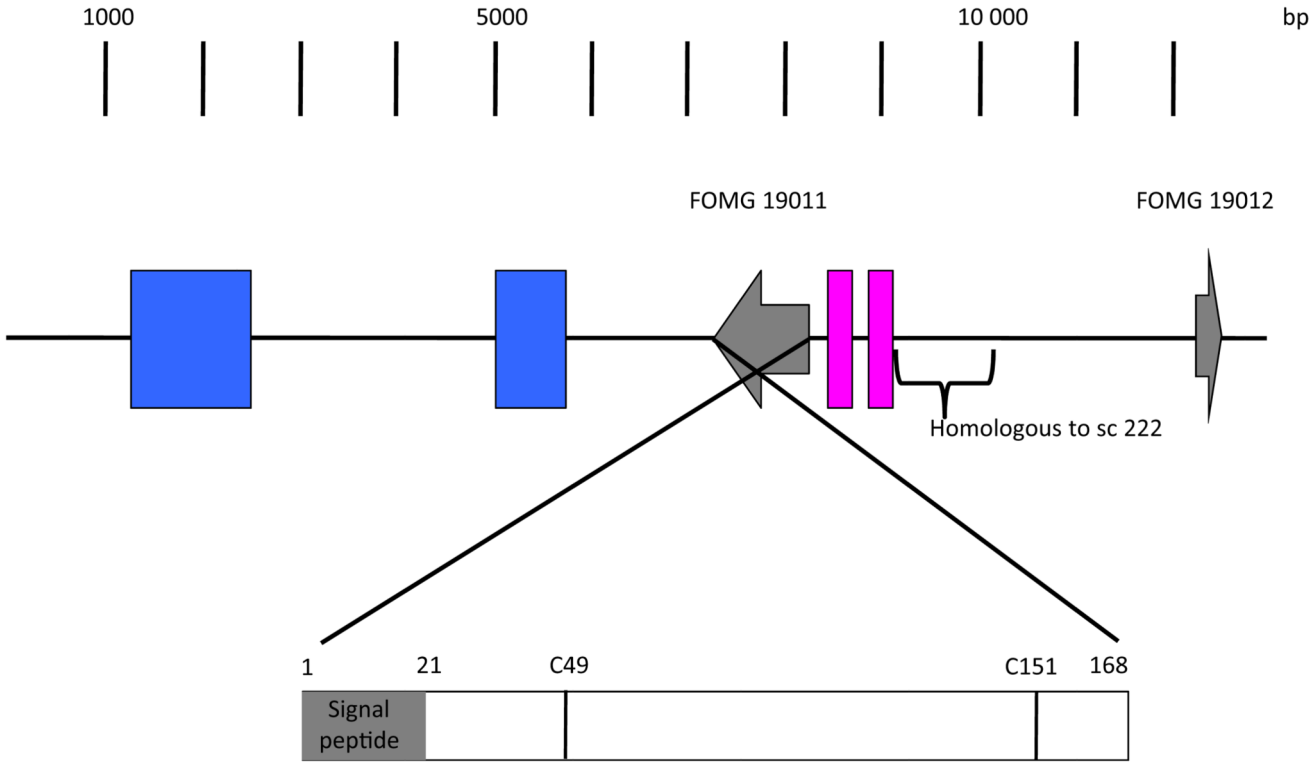

Fig. 4.

The *AVRFOM2* locus and protein *of Fusarium oxysporum* f. sp. *melonis* isolate Fom001. Schematic representation of the supercontig 236 harbouring *AVRFOM2* (FOXG_19011) of Fom001. Blue boxes indicate transposable elements. Pink boxes represent Miniature Impala (mimp) transposable elements. Contig size is indicated at the top.

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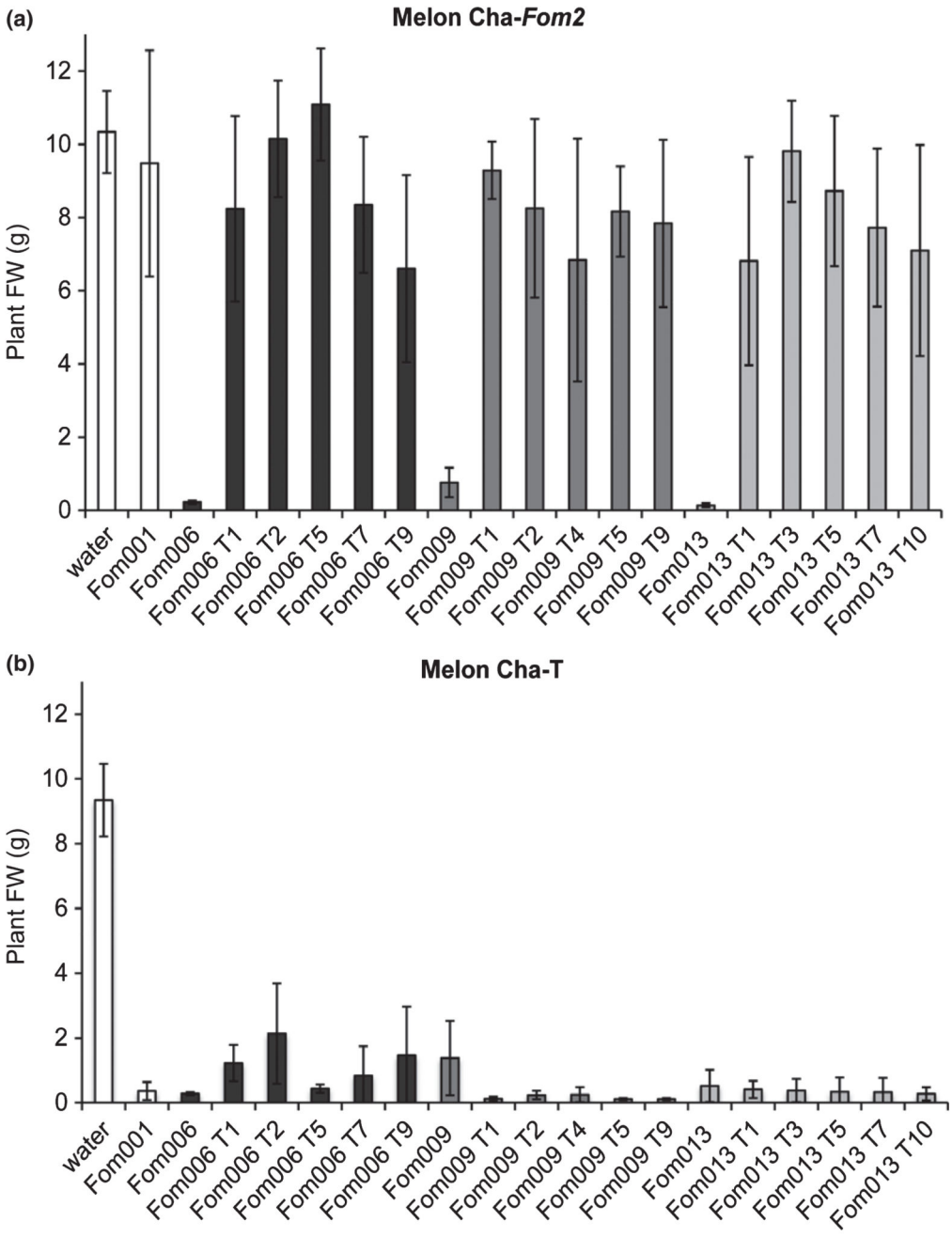

Fig. 5.

Genetic complementation of three different *Fusarium oxysporum* f. sp. *melonis* (*Fom*) race 2 isolates with *AVRFOM2* results in *Fom-2*-mediated resistance of melon. We cloned *AVRFOM2* with its own promoter (1 kb upstream of the start codon) and its own terminator (1 kb downstream of the stop codon) and ectopically inserted it in the genomes of three different race 2 isolates (Fom006, Fom009, Fom013) via Agrobacterium-mediated transformation. Ten-day-old melon seedlings with (Cha-Fom2) or without (Cha-T) the *Fom-2* resistance gene were inoculated with conidia of five independent strains that were

genetically complemented with *AVRFOM2* for each isolate as well as with conidia of the wild-type race 2 strains and Fom001 (race 1). Twelve days after inoculation we determined the plant fresh weight (FW). (a) High plant FW of Cha-*Fom-2* plants indicates resistance of the plant to the fungal infection. (b) Low FW of Cha-T plants indicates that the fungal strains are able to cause infection in the absence of *Fom-2*. Error bars indicate ± SD ($n = 5$ for water, Fom001, Fom006, Fom009, Fom013 or $n = 8$ for genetic complements). The experiment was repeated twice with similar results.

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Table 1

Fusarium oxysporum f. sp. *melonis* (*Fom*) strains used in this study

Isolate	Original designation	Race	VCG	Origin of isolate	Reference
Fom001	NRRL26406	1	0136	Mexico	O'Donnell *et al.* (1998a); Lievens *et al.* (2009); www.broadinstitute.org
Fom004	Fom0122	0	0134	Southern Spain	Alvarez *et al.* (2005)
Fom005	Fom0123	1	0134	Southern Spain	Alvarez *et al.* (2005)
Fom006	Fom0124	2	0134	Southern Spain	Alvarez *et al.* (2005)
Fom009	–	2	0135	Israel	Herman & Perl-Treves (2007)
Fom010	–	1	–	Israel	Herman & Perl-Treves (2007)
Fom011	–	0	–	Israel	Herman & Perl-Treves (2007)
Fom012	ML2	0	0134	–	Perchepied & Pitrat (2004)
Fom013	–	2	0134	Spain	Perchepied *et al.* (2005)
Fom016	Fom26	1	0134	–	Perchepied *et al.* (2005)

Fom isolates were obtained from different origins. Race and vegetative compatibility group (VCG) testing was performed in our laboratory.

Table 2

Effector candidates of *Fusarium oxysporum* f. sp. *melonis* isolate Fom001 identified by presence of an upstream Miniature Impala (mimp)

Effector candidate	FOMG number	Length (aa)	Distance mimp IR-ATG (bp)	No. of copies in Fom001 genome[1]	Homologues in other *Fo formae speciales*[2]	Homologues in other fungi[3]
1A	FOMG_18653	169	650	1	Yes	None
1B	FOMG_19455	166	818	1	Yes	None
2	FOMG_19011	167	689	1	No	None
3	FOMG_19769	110	267	1	No	*Colletotrichum gloeosporioides*, *Colletotrichum fioriniae*
4	not annotated	64	778	2	Yes	*Fusarium fujikuroi*
5	not annotated	161	416	2	Yes	None
6	FOMG_19741 (wrong gene model)	265	817	2	Yes	None
7	FOMG_19453 (wrong gene model-intron right after ATG)	148	1975	1	No	*Colletotrichum orbiculare*
8	FOMG_19621 (wrong gene model – 3 introns – too long)	112	1302	2	Yes	none
FomSIX6	FOMG_18978	145	433	1	Yes	*Colletotrichum fioriniae*, *Colletotrichum orbiculare*, *Colletotrichum higginsianum*
LysM	FOMG_19260 (shares promoter with 8)	165	705	1	Yes	*Leptosphaeria maculans*, *Colletotrichum gloeosporioides*, *Colletotrichum orbiculare*, *Bipolaris victoriae*, *Bipolaris sorokiniana*, *Bipolaris zeicola*, *Marssonina brunnea* f. sp. *multigermtubi*, *Setosphaeria turcica*, *Verticillium dahliae*

[1] Estimation based on genome read depth per base.

[2] Based on BlastN searches against Fusarium Comparative genomic sequence at http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/Blast.html.

[3] Based on BlastP searches against NCBI nonredundant database. Hits are reported as homologues if > 50% of the query is covered and the amino acid identity is > 40%. IR, inverted repeats.