The FonSIX6 gene acts as an avirulence effector in the Fusarium oxysporum f. sp. niveum - watermelon pathosystem

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When infecting a host plant, the fungus Fusarium oxysporum secretes several effector proteins into the xylem tissue to promote virulence. However, in a host plant with an innate immune system involving analogous resistance proteins, the fungus effector proteins may trigger resistance, rather than promoting virulence. Identity of the effector genes of Fusarium oxysporum f. sp. niveum (Fon) races that affect watermelon (Citrullus lanatus) are currently unknown. In this study, the SIX6 (secreted in xylem protein 6) gene was identified in Fon races 0 and 1 but not in the more virulent Fon race 2. Disrupting the FonSIX6 gene in Fon race 1 did not affect the sporulation or growth rate of the fungus but significantly enhanced Fon virulence in watermelon, suggesting that the mutant ΔFon1SIX6 protein allowed evasion of R protein-mediated host resistance. Complementation of the wild-type race 2 (which lacks FonSIX6) with FonSIX6 reduced its virulence. These results provide evidence supporting the hypothesis that FonSIX6 is an avirulence gene. The identification of FonSix6 as an avirulence factor may be a first step in understanding the mechanisms of Fon virulence and resistance in watermelon and further elucidating the role of Six6 in Fusarium-plant interactions.

Watermelon [Citrullus lanatus (Tunb.) Matsum. & Nakai] is an important cucurbit crop accounting for 7% of the agricultural land area devoted to vegetable production worldwide. The total annual production of watermelon is approximately 90 million tons, making it among the top five most consumed fresh fruits (http://faostat.fao.org). Fusarium wilt, caused by the soil-borne fungus Fusarium oxysporum f. sp. niveum (Fon), is a major disease of watermelon throughout the world, with a large adverse impact on watermelon yield and quality.

There are three common physiological races (0, 1 and 2) of this pathogen, classified according to their reactions with differential watermelon genotypes (Table 1)¹⁻⁴. Race 0 is pathogenic only in watermelon cultivars with no resistance genes. Race 1 is the predominant race throughout commercial watermelon regions worldwide, and several watermelon cultivars, such as cv. Calhoun Gray, are resistant to this race. Race 2 is highly aggressive to all current commercial watermelon cultivars and hybrids. Race 3, the most virulent race of Fon described to date, was shown to cause over 90% wilt on PI296341-PR, whereas no disease was caused by a race 2 isolate.

The co-evolution of plants and microorganisms involves complex mechanisms of attack and defence, implicating the innate immune system of plants and virulence factors of pathogens.² The first layer of plant defence, known as basal immunity, is based on the recognition of conserved microbial molecules but can be suppressed by microbial virulence factors known as “effectors”. Plants respond to this suppression by employing a second layer of defence, resistance (R) gene-based immunity, which relies on the recognition of effectors.² Finally, the pathogen evolves further and escapes detection by the R gene product by eliminating the detected virulence factor or by suppressing the defence induced by R gene products.³ Effectors may be defined as pathogen proteins and small molecules that alter host-cell structure and function. These alterations either facilitate infection (virulence factors and toxins) or trigger defence responses (avirulence factors and elicitors), or both.⁴

The secreted effector proteins of F. oxysporum f. sp. lycopersici (Fol) infecting tomato have been identified through proteomic analysis of xylem sap from tomato plants infected with Fol. These proteins have been designated the Six (secreted in xylem) proteins and include Six1 to Six7.⁷⁻¹¹ Several functions of the Six proteins

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have been identified thus far. Avr3 (Six1) is required for I-3-mediated resistance and Avr1 (Six4) is required for I-mediated resistance. Additionally, both proteins have functions other than triggering avirulence: Avr3 is required for full virulence, whereas Avr1 suppresses I-2- and I-3-mediated disease resistance. Subsequently, Avr2 (Six3) shows both activities: it is required for full virulence in susceptible tomato host plants while triggering resistance in plants carrying the resistance gene I-2. Six5 is required for full virulence in susceptible plants, and knockout of this gene can breach I-2-mediated disease resistance. Avr2 and Six5 interact in yeast two-hybrid assays as well as in planta. The AVR2-SIX5 gene pair is required to activate I-2-mediated immunity in tomato, while Six6 is a true effector that enhances virulence and simultaneously suppresses I-2-mediated cell death. Screening of effector proteins indicated that the three AVR gene sequences (AVR1, AVR2, and AVR3) and the SIX5 gene sequence are not present in the Fon races, while the SIX6 gene homologue is present, although its biological function has not been determined.

In this study, we identified and analysed the biological function of the SIX6 gene in Fon (FonSIX6) and demonstrated that FonSIX6 is an AVR gene playing a key role in the Fon-watermelon pathosystem.

### Results

**Cloning and analysis of FonSIX6 and flanking sequences.** Using the genome sequence of Fol (http://www.broadinstitute.org/) as a reference for constructing PCR primers, we cloned a SIX6 gene of Fon. Here, we used primers that annealed immediately outside the FolSIX6 gene ORF. The resultant gene was designated FonSIX6. Genome searches using the FonSIX6 ORF sequence as a query showed high sequence homology of SIX6 with F. oxysporum f. sp. melonis (Fom, 100%) and Fol (94.91%) (Supplementary Fig. S1). To obtain additional information about FonSIX6, the Fol genome was used as a reference for designing specific PCR primers for the flanking sequence. However, no PCR fragments were amplified, suggesting that the Fol and Fon genomic sequences are different. Finally, the 1974 bp upstream sequence (directly adjacent to the start codon, −1974 bp) and the 453 bp downstream (directly 3′ to the stop codon, +453 bp) of the FonSIX6 open reading frame were cloned via chromosome walking (Supplementary Fig. S2).

**Analysis of the conserved FonSIX6 homologue sequence in a watermelon-infecting strain.** The deduced FonSIX6 protein consists of 215 amino acids and contains 8 cysteine residues (Fig. 1). This intronless gene is predicted to encode a 21.85 kDa mature protein (after cleavage of the predicted N-terminal signal peptide) (http://web.expasy.org/compute_pi/). Amino acid sequence comparisons between FonSIX6 and FolSIX6 (NCBI GenBank:ACN69116.1) showed 90.23% identity, with minor differences. Using the SignalP 4.0 Server, the FonSIX6 protein was predicted to contain a signal peptide consisting of 16 amino acids at its N-terminus.

**Impact of FonSIX6 disruption on fungal development.** To determine whether FonSIX6 affects fungal growth and microsclerotia production in Fon, the growth patterns of the gene disruption mutant ΔFonSIX6 on potato dextrose agar (PDA), minimal medium (MM) and complete medium (CM) media were compared with those of the wild-type Fon strain and the gene-complemented ΔFon1SIX6 + SIX6 and Fon2 + SIX6 strains.

During growth on PDA, the Fon mycelium produced red-brown pigments, whereas no red-brown colour was observed on MM or CM medium. Measurement of colony diameter on PDA, MM, or CM medium during the first 4 days of culture indicated that the radial growth of the FonSIX6-disrupted and complemented strains did not differ from that of the wild-type strain (Fig. 3). The morphology and quantity of spores also did not differ substantially, as observed under a microscope. These observations indicate that the FonSIX6 gene is not essential for Fon growth and development.

**FonSIX6 is expressed 3 days after Fon infection.** To determine whether FonSIX6 was expressed during earlier stages of infection, RNA was collected from Fon-infected watermelon roots on the 1st, 2nd, 3rd, 4th and 5th day post-inoculation (DPI). The expression of FonSIX6 was monitored using reverse-transcriptase polymerase chain reaction (RT-PCR). FonSIX6 transcripts could be detected on the 3rd through the 5th DPI in infected plants, whereas the mock-inoculated controls did not produce this transcript (Fig. 4).

**Watermelon inoculation with wild-type and transformants.** To determine the role of FonSIX6 in the infection of watermelon, FonSIX6 knockout mutants (ΔFonSIX6) were generated in Fon race 1 by replacing FonSIX6 with a hygromycin resistance cassette (Fig. 5a). Then, the virulence of the ΔFonSIX6 strain was assessed by inoculating watermelon seedlings (cv. Calhoun Gray, resistant to Fon 1). To our surprise, the severity of disease

| Cultivar or genotype | Race 0 | Race 1 | Race 2 |
|----------------------|-------|-------|-------|
| Sugar Baby           | S     | S     | S     |
| Charleston Gray      | R     | S     | S     |
| Calhoun Gray         | R     | R     | S     |
| PI 296341-FR         | R     | R     | R     |

Table 1. Watermelon genotypes used to differentiate races of Fusarium oxysporum f. sp. niveum.

*S = susceptible. R = resistant.*
of *F. oxysporum* horizontal transfer from another species, leading to the hypothesis that horizontal chromosome transfer in sub-regions enriched for DNA transposons. The LS genome regions could have been acquired through (chromosome 14), also known as the pathogenicity chromosome, and are associated with chromosomal commercial watermelon cultivars and hybrids and is clearly a distinct race2. Here, the association of might be strains of race 1 with varying aggressiveness. On the other hand, race 2 is highly aggressive to all current the distinction between race 0 and race 1 may be more quantitative than qualitative. Consequently, races 0 and 1 watermelon genotypes2–4. This study indicates the possibility that the races (0, 1 and 2) of the *F. oxysporum* f. sp. *lycopersici* gene with the pathogenicity of different gene with the pathogenicity of different is gradually increased in races 0, 1 and 2, there is growing speculation that races may provide a potential cultivar-specific pathogenicity marker for *F. oxysporum* f. sp. *lycopersici*. In *FolSIX6*: the six gene from *F. oxysporum* f. sp. *lycopersici* (ACY39286.1).

The vascular pathogen *F. oxysporum* is an asexual fungus with a broad host range that causes wilt and root diseases in many economically important crop plants, including watermelon19. In *Fol*: 14 'Secreted in xylem' (Six) proteins (Six1~14) have been identified from *F. oxysporum* f. sp. *lycopersici* 10,20. Of the Six proteins, Six6 contributes to virulence and suppresses I-2-mediated cell death. Although a Six homologue sequence has been identified in *Fol* isolates, its functional has not been characterized17,18. In this report, we describe the identification and functional analysis of the SIX6 gene of *Fol*.

Earlier studies showed that the *FolSIX6* gene was present in the *forma specialis niveum* in isolates 546 and 704 but not in isolates 703, 705, CBS 187.60, CBS 418.90, and CBS 419.9011,18. Three generally accepted physiological races (0, 1 and 2) of the *Fol* pathogen have been identified to date, according to their effects in differential *F. oxysporum* f. sp. *niveum* infection10,20. Of the Six proteins, Six6 contributes to virulence and suppresses I-2-mediated cell death. Although a Six homologue sequence has been identified in *Fol* isolates, its functional has not been characterized17,18. In this report, we describe the identification and functional analysis of the SIX6 gene of *Fol*.

### Discussion

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Although the pathogenicity of *Fol* is gradually increased in races 0, 1 and 2, there is growing speculation that the distinction between race 0 and race 1 may be more quantitative than qualitative. Consequently, races 0 and 1 might be strains of race 1 with varying aggressiveness. On the other hand, race 2 is highly aggressive to all current commercial watermelon cultivars and hybrids and is clearly a distinct race3. Here, the association of the *FolSIX6* gene with the pathogenicity of different *Fol* races may provide a potential cultivar-specific pathogenicity marker that is useful for defining host targets and evolutionary bottlenecks that control the *Fol*-watermelon pathosystem.

In *Fol*, most SIX genes are located in the same lineage-specific (LS) genomic region- chromosome (chromosome 14), also known as the pathogenicity chromosome, and are associated with chromosomal sub-regions enriched for DNA transposons. The LS genome regions could have been acquired through horizontal transfer from another species, leading to the hypothesis that horizontal chromosome transfer in *F. oxysporum* can generate new pathogenic lineages21,22. *FolSIX6* is located on a supernumerary chromosome 14, an LS chromosome21. Searches carried out using the *FolSIX6* ORF sequence as a query showed strong sequence identity to SIX6 of *Fon* (100%) and *Fol* (94.91%) (Supplementary Fig. S1). Comparison of the *FolSIX6* ORF and...
its flanking sequences between the Fon and Fol genomes in the –609 ~ +157 and –433 ~ +157 regions showed nucleotide sequence identities of 99.58% and 96.29%, respectively (Supplementary Fig. S2). The genome of F.

Figure 3. Colony growth of deletion and complementation mutants compared with wild-type Fon on PDA, CM, and MM. Fon1: Fusarium oxysporum f. sp. niveum wild race 1; Fon2: Fusarium oxysporum f. sp. niveum wild race 2; ΔFon1SIX6: race 1 with SIX6 disrupted by gene replacement; ΔFon1SIX6 + SIX6: ΔFon1SIX6 transformed with SIX6; Fon2 + SIX6: race 2 transformed with SIX6; PDA: potato dextrose agar medium; MM: minimal medium; CM: complete medium. The photographs were taken from the top and bottom of the plates 4 days after incubation.

Figure 4. FonSIX6 is expressed during the early stages of infection. Reverse-transcriptase polymerase chain reaction analysis of watermelon actin (ACTIN) or FonSIX6 expression using RNA isolated from the roots of watermelon seedlings, which were either mock or Fusarium oxysporum f. sp. niveum inoculated and harvested on the 1st, 2nd, 3rd, 4th and 5th day post-inoculation (DPI). Water was included as a negative control (−), while genomic DNA from Fusarium oxysporum f. sp. niveum (+) was used as a positive control.
oxysporum 4287 (FO2) has been sequenced and is available (http://www.broadinstitute.org/). BLAST searches of this genome sequence database using the FonSIX6 sequence segment (−1974 ~ +453) as a query showed the presence of high sequence identity (90 ~ 100%) in 5 distinct segments. Segment 1 (−1974 ~ −686) is located in supercontig 37 of Chr15 (91.47% nucleotide sequence identity). Segments 2 ~ 4 are located on supercontig 22 of Chr14 at different positions with various nucleotide sequence identities, with segment 2 (−685 ~ −654) showing 100% identity, segment 3 (−653 ~ −434) showing 95% identity and the segment 4 (−433 ~ +157) showing 96.29% identity, while segment 5 (+158 ~ +453), located on nonpositional scaffolds, exhibited 98.31% nucleotide sequence identity. These analyses suggested that the −433 ~ +157 sequence segment identified in Fon may contain the full gene sequences that are necessary to complete the function of FonSIX6.

Here, we generated FonSIX6 gene knockout mutants in race 1, evaluated the complementation of the knockout mutants and wild-type race 2 with the FonSIX6 gene, and observed that the disruption of FonSIX6 did not affect the growth rate or fungal sporulation. These results demonstrate that FonSIX6 is not absolutely necessary for Fon growth and development. Therefore, the change in the virulence of ΔFonSIX6 is not associated with fungal growth or development and is instead due to the effector’s key role in the Fon-watermelon pathosystem.

In comparison with the watermelon plants inoculated with wild-type Fon, the severity of the disease symptoms of the watermelon plants inoculated with the ΔFonSIX6 transformants was significantly enhanced. These results suggest that the mutant ΔFonSIX6 protein allowed evasion of R protein-mediated host resistance. On the other hand, complementation of wild-type Fon 2 (lacking FonSIX6) with the FonSIX6 gene reduced its virulence. Taken together, these results indicate that FonSIX6 is an AVR gene. Loss of function of an AVR gene (FonSIX6) in race 2 allowed the pathogen to avoid the induction of resistance in a watermelon cultivar. Thus, the pathogen gained pathogenicity in that cultivar, and a new pathogenic race (race 2) emerged. The three known races
(1, 2 and 3) carry AVR genes in different combinations in Fol. Fol race 2 emerged from race 1 by losing AVR1 and thereby allowed evasion of host resistance mediated by I (the resistance gene corresponding to AVR1). Race 3 emerged when race 2 sustained a point mutation in AVR2, allowing it to evade I-2-mediated resistance of the host15,23. The results of the present study indicate that Fon race 2 may have emerged from race 1 owing to loss of the entire FonSIX6 gene sequence or may have resulted from a mutation that impaired the function of the FonSIX6 gene, evading mediated host resistance. Additional studies are needed to further determine the differences between Fon races 1 and 2.

Methods
Alignment. DNA sequence and protein alignments were performed using the computer programs ClustalW and DNAMAN. The genome of Fol (http://www.broadinstitute.org) was used as a reference sequence for constructing PCR primers to clone the homologous SIX6 gene sequence of Fon.

Fon races and mutational lines used in this study. The following Fon strains were used: Fon 0 (race 0), Fon 1 (race 1), and Fon 2 (race 2) (a kind gift from the National Engineering Research Center for Vegetables, Beijing, China). ΔFonSIX6 was race 1 with SIX6 disrupted by gene replacement. ΔFonSIX6 + SIX6 was ΔFonSIX6 transformed with SIX6. Fon2 + SIX6 was race 2 transformed with SIX6.

FonSIX6 disruption and complementation constructs. The FonSIX6 flanking sequence was cloned using the Genome Walker Universal kit (Clontech). The FonSIX6 disruption construct was generated via PCR amplification of FonSIX6 upstream and downstream sequences (with partial FonSIX6 sequences) for homologous recombination, followed by insertion in front of and behind the hygromycin resistance gene in the vector pHxH24. An upstream fragment, from 1560 to 252 bp upstream of the start codon, was cloned into pDH2 between the EcoRI 1 (′ > CCAGTTACAGCTCTGTATGCTGTCT < 3′) and Sac I 1 (′ > CGAGCTCGTGCATGTAATGTTGTTT < 3′) sites, and a downstream fragment, from 212 bp after the start codon to 1099 bp downstream of the stop codon, was cloned into pDH12 between the Sac 1 1 (′ > CGGTCTCTGACCGCTCGTCTGTA < 3′) and Xba I 1 (′ > TCTCGTCTAGAATCGACGCGGCTGTAAGGAT < 3′) sites (Fig. 5a). Transformants were selected on hygromycin B and confirmed by PCR (Supplementary Fig. S3).

To generate a FonSIX6 complementation construct, a fragment of 2659 bp containing the FonSIX6 open reading frame, 1560 bp of upstream sequence and 451 bp of downstream sequence was amplified via PCR using primers with EcoRI 1 and Xba I linkers (underlined) (′ > CCAGTTACAGCTCTGTATGCTGTCT < 3′ and 5′ > TCTCGTCTAGAATCGACGCGGCTGTAAGGAT < 3′). This fragment was cloned into pCOM24. Transformants were selected on geneticin and confirmed through PCR (Supplementary Figs S4 and S5). Transformation of the constructs into Fon was carried out using Agrobacterium as described previously25.

Plant material and fungal strains. The following watermelon differentials were used: cv. Sugar Baby and cv. Calhoun Gray. Fon race 0 causes wilt in cv. Sugar Baby; Fon race 1 causes wilt in cv. Sugar Baby and cv. Charleston Gray but not in cv. Calhoun Gray; and Fon race 2 causes wilt in all of the differential cultivars but not in PI296341-FR3.

Pathogenicity assay. Each Fon isolate was cultured on potato sucrose broth (PSB) for 5 days at 25 °C at 120 rpm, and conidial suspensions (1.0 × 109 conidia ml−1) were prepared.

Seeds of each cultivar were sown in vermiculite in plastic pots (6 by 6 by 5 cm, 32 cells tray−1) and grown in a greenhouse set at 24–30 °C on top of a heat pad (30 °C). The standard root dip method was used to inoculate watermelon seedlings. At the first true leaf stage, the seedlings were dipped in a conidial suspension (1.0 × 106 conidia ml−1) for 5 min and replanted to a vermiculite tray. Disease was scored at 15 days post-inoculation. The disease assay results were quantified based on the average plant weight and the typical disease symptoms of yellowing, stunting and wilting. Because the inoculation methods involving direct dipping or root cutting yielded a similar disease incidence and symptom severity, the data obtained using the two methods were combined for analysis. All of the tests were repeated at least three times.

RNA isolation and RT-PCR. For FonSIX6 gene expression analysis, RT-PCR experiments were performed using tissue harvested from Fon1-infected roots of watermelon cv. Sugar baby. The root samples were ground in liquid nitrogen. Then, total RNA was extracted with the RNAiso plus reagent (Takara), and DNA was removed with recombinant DNase I (Takara). cDNA was subsequently synthesized using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara). The primer combinations w-actinF/w-actinR (W-actinF: 5′ > AATACCGTCTGATATGTCG < 3′; W-actinR: 5′ > GATGGAGTTGTTAGTGTTTAGTTTG < 3′) and FonSIX6F/FonSIX6R (FonSIX6F: 5′ > CGGTCTCTAGAATCGACGCGGCTGTAAGGAT < 3′; FonSIX6R: 5′ > GGTGTTGACGCGGCTGTAAGGAT < 3′) were used to amplify the watermelon actin gene and FonSIX6.

Vegetative growth, conidiation and microsclerotia formation assays. For each sample, a 0.5 μL drop of a conidial suspension (1.0 × 109 conidia ml−1) was inoculated onto the centre of a 90-mm Petri dish containing potato dextrose agar (PDA), minimal medium (MM), and complete medium (CM)16 and cultured at 25 °C. The colony diameter and morphology of the vegetative mycelia were examined at 4 days after inoculation. To estimate conidial production, discs of 7 mm in diameter obtained from the edge of a 10 day-old fungal colony on PDA medium were suspended in 1 mL sterilized water, then subjected to shaking at 150 rpm for 10 min. A 100 μL drop of the conidial suspension was subsequently placed onto a haemocytometer, and the spores were counted under a microscope. All of the tests were repeated at least three times.
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Author Contributions

X.N. and M.F. conceived the experiments. X.N., X.Z. and Y.S. conducted the experiments. X.N., K.-S.L. and A.L. analysed the results and wrote the manuscript. All authors reviewed the manuscript.

Additional Information

Accession codes: Sequence data for FonSIX6 with flanking sequences from this paper have been deposited with the EMBL/DBJ/GenBank data libraries under accession no. LT160066.1.

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