A Highly Conserved Effector in *Fusarium oxysporum* Is Required for Full Virulence on *Arabidopsis*

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Submitted 16 August 2011. Accepted 21 September 2011.

Secreted-in-xylem (SIX) proteins of the vascular wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici* are secreted during infection of tomato and function in virulence or avirulence. *F. oxysporum* formae speciales have specific host ranges but the roles of SIX proteins in diverse hosts are unknown. We identified homologs of *F. oxysporum* f. sp. *lycopersici* SIX1, SIX4, SIX8, and SIX9 in the genome of *Arabidopsis* infecting isolate Fo5176. A SIX4 homolog (termed Fo5176-SIX4) differed from that of *F. oxysporum* f. sp. *lycopersici* (Fol-SIX4) by only two amino acids, and its expression was induced during infection of *Arabidopsis*. Transgenic *Arabidopsis* plants constitutively expressing Fo5176-SIX4 had increased disease symptoms with Fo5176. Conversely, Fo5176-SIX4 gene knock-out mutants ($\Delta six4$) had significantly reduced virulence on *Arabidopsis*, and this was associated with reduced fungal biomass and host jasmonate-mediated gene expression, the latter known to be essential for host symptom development. Full virulence was restored by complementation of $\Delta six4$ mutants with either Fo5176-SIX4 or Fol-SIX4. Thus, Fo5176-SIX4 contributes quantitatively to virulence on *Arabidopsis* whereas, in tomato, Fol-SIX4 acts in host specificity as both an avirulence protein and a suppressor of other race-specific resistances. The strong sequence conservation for SIX4 in *F. oxysporum* f. sp. *lycopersici* and Fo5176 suggests a recent common origin.

To protect themselves from pathogens, plants employ multiple defenses acquired during plant–pathogen coevolution. A plant's first defensive front against microbial attack consists of nonspecific preformed physical and chemical barriers that restrict pathogen entry. In addition to these barriers, plants have an immune response that relies on the detection of generic pathogen-derived elicitors known as pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (Bent and Mackey 2007; Boller and He 2009; Gohre and Robatzek 2008). Fungal cell wall components such as chitin, glucan, or glycoprotein and bacterial components such as lipopolysaccharide, elongation factor-Tu, or flagellin can all act as PAMPs. A PAMP-triggered defense response, termed PAMP-triggered immunity (PTI), usually halts or restricts the infection process; however, some microbes have adapted to this pressure by evolving effector proteins that interfere with or suppress the plant's defense response (Jones and Dangl

2006). In response to this, plants have evolved specific receptors broadly referred to as resistance (R) proteins that directly or indirectly recognize microbial effectors and trigger a second layer of defense termed effector-triggered immunity (ETI). Pathogens can avoid ETI and cause disease by eliminating or modifying the effector recognized by the plant's surveillance system. This co-evolutionary process between plants and microbial pathogens, originally proposed by Jones and Dangl (2006), has been reviewed extensively in recent years (Anderson et al. 2010; Bent and Mackey 2007; Boller and He 2009; Chisholm et al. 2006).

Much of our understanding of PTI and ETI has come from bacterial effectors (Gohre and Robatzek 2008). Like bacteria, fungal pathogens also secrete effectors, some of which have functions in virulence and others that are directly or indirectly recognized by R proteins and trigger immunity (Stergiopoulos and de Wit 2009). Usually, there is only a low degree of amino acid sequence conservation among fungal effectors across fungal lineages but most are thought to be secreted, cysteine-rich proteins. Although, potentially, a large number of effectors are produced during plant–fungus interactions, thus far, a function in virulence has only been determined for a few fungal effectors. For example, some effectors exhibit chitin-binding activity for protection from plant chitinases, or function as protease inhibitors (Stergiopoulos and de Wit 2009; Van De Wouw and Howlett 2011).

The fungal pathogen *Fusarium oxysporum* is responsible for vascular wilt disease, with pathogenic isolates classified into more than 120 formae speciales based on the individual plant species they infect. The infection process has been studied in detail in tomato (Michielse and Rep 2009) where, following germination of spores in the soil, hyphae colonize the root surface and directly penetrate the root epidermal layer, apparently at random sites, and advance through the root cortex intercellularly where they reach the xylem vessels. As a result of fungal colonization and host defenses attempting to limit pathogen spread, vessels become clogged, leading to water stress, collapse of vascular tissues, necrosis, and eventual plant death. Infection processes for strains of *F. oxysporum* infecting *Arabidopsis* differ slightly in that primary and lateral root initials appear to be a preferential site of initial fungal colonization and penetration (Czymmek et al. 2007; Kidd et al. 2011) and wilting and collapse of the foliar tissue is preceded by leaf chlorosis.

Four R genes of tomato conditioning resistance against *F. oxysporum* f. sp. *lycopersici* have been identified in wild tomato relatives and introgressed into commercial tomato cultivars (Takken and Rep 2010). These are termed immunity (I) genes *I*, *I-1*, *I-2*, and *I-3*. Thus far, only *I-2* has been cloned and encodes a nucleotide-binding leucine-rich repeat protein (Simons et al. 1998). Corresponding avirulence (*Avr*) genes in *F. oxysporum* f.

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sp. *lycopersici* have been identified by reverse genetic analysis of genes encoding fungal proteins recovered from xylem sap of infected tomato plants, where they are termed secreted-in-xylem or (SIX) proteins (Rep et al. 2002, 2004; Takken and Rep 2010). SIX4 or Avr1 is the Avr gene product recognized by I and I-1. Similarly, SIX3 (also known as Avr2) and SIX1 (also known as Avr3) are recognized by I-2 and I-3, respectively. Interestingly, the tomato *R* gene *I-2* is expressed in cells associated with vascular tissues (Mes et al. 1999, 2000) where SIX proteins are secreted by the fungus. The cloning of the *F. oxysporum* avirulence *SIX3/Avr2* (Houterman et al. 2009) along with the cognate *R* gene (*I-2*) from tomato (Simons et al. 1998) represented the first complete Avr-*R* gene pair molecularly characterized for a *Fusarium* pathogen and any root-infecting or xylem-colonizing fungus.

Some of the SIX proteins have been shown to have roles in both virulence and avirulence in a manner consistent with the PTI and ETI model for host-pathogen co-evolution (Jones and Dangl 2006) discussed above. Both *SIX3/Avr2* (Houterman et al. 2009) and *SIX1/Avr3* (Rep et al. 2004) are required for virulence on a fully susceptible tomato genotype. In contrast, *SIX4/Avr1* appears not to be required for virulence on susceptible tomato and is dispensable and absent from the published *F. oxysporum* f. sp. *lycopersici* reference genome sequence (Ma et al. 2010). However, *SIX4/Avr1* is required for virulence on tomato genotypes carrying either the *I-2* or *I-3* gene, where *SIX4* appears to suppress their resistance function (Houterman et al. 2008). Therefore, it is believed that the acquisition of *SIX4/Avr1* has enabled *F. oxysporum* f. sp. *lycopersici* to overcome the resistance conferred by *I-2* and *I-3* without the penalty on virulence that would have resulted through the alternative of losing *SIX3/Avr2* and *SIX1/Avr3* (Takken and Rep 2010). The source of the *SIX4/Avr1* gene in *F. oxysporum* f. sp. *lycopersici* is not known but genome analysis of *F. oxysporum* f. sp. *lycopersici* has shown that other *SIX* effector genes are primarily encoded on supernumerary “pathogenicity-associated” chromosomes which can transfer between strains (Ma et al. 2010), facilitating such gene acquisition by horizontal chromosome transfer events (Mehrabi et al. 2011).

The *Arabidopsis*–*F. oxysporum* interaction is also developing as a model system to study host–*F. oxysporum* interactions, with some strains that cause disease on *Brassica* spp. also being able to infect *Arabidopsis* (Campbell et al. 2003; Diener and Ausubel 2005; Edgar et al. 2006). In some instances, these strains have been assigned to formae speciales including *F. oxysporum* f. sp. *conglutinans*, *matthiolae*, and *raphani*, which are pathogenic on cabbage, stock, and radish, respectively (Diener and Ausubel 2005). Most research on the *Arabidopsis*–*F. oxysporum* interaction has focused on the host reaction to infection and underpinning molecular mechanisms due to the large array of available genetic resources in *Arabidopsis*. Although the manipulation of key downstream signaling components such as hormone receptors, transcription factors, and defense genes can provide varying degrees of resistance to *F. oxysporum* in *Arabidopsis* (Anderson et al. 2004; Berrocal-Lobo and Molina 2004; Eppe et al. 1997; Kidd et al. 2009; McGrath et al. 2005; Thatcher et al. 2009), no *R* genes in a classical sense have been found. Variation in ecotype susceptibility and resistance has been identified (Czymmek et al. 2007; Diener and Ausubel 2005) (K. Kazan, unpublished) but, thus far, these differences appear polygenic in nature (Diener and Ausubel 2005). For example, six quantitative trait loci were identified in the Col-0 ecotype, with the single gene *resistance to F. oxysporum 1 (RFO1)* being the largest contributor to resistance (Diener and Ausubel 2005). *RFO1* encodes a wall-associated kinase-like kinase and enhances resistance nonspecifically to several *Brassica* spp.-infecting *F. oxysporum* formae speciales.

In total, 11 *SIX* proteins have been identified thus far in *F. oxysporum* f. sp. *lycopersici*, and the function of most of these remains unknown (Houterman et al. 2007; Lievens et al. 2009; Ma et al. 2010). It has been shown that *SIX* genes are also present in formae speciales other than *F. oxysporum* f. sp. *lycopersici*; for example, *SIX6* and/or *SIX7* homologs are present in *F. oxysporum* f. sp. *lilii*, *melonis*, *radicis-cucumerinum*, and *vasinfectum* (Chakrabarti et al. 2011; Lievens et al. 2009). However, the possible role in pathogenicity of *SIX* genes outside of *F. oxysporum* f. sp. *lycopersici* has not been investigated. Assessments of *SIX* gene diversity and occurrence have been based on polymerase chain reaction (PCR) analysis, which may not always identify homologous sequences in other fungi. Using new short-read sequencing techniques, we sequenced the genome of the *F. oxysporum* isolate Fo5176 (Ma et al. 2010, supplementary data), which is virulent on *Arabidopsis* but does not cause disease on tomato (M. Rep, personal communication). Genome sequence mining facilitates the identification of *SIX* gene homologs and, here, we report the presence of *SIX1*, *SIX4*, *SIX8*, and *SIX9* homologs in the genome of the *Arabidopsis* infecting *F. oxysporum* isolate Fo5176. Even though the Fo5176 strain was isolated from infected *Brassica oleracea* in Australia and the strain Fol004 of *F. oxysporum* f. sp. *lycopersici* containing the *SIX4/Avr1* gene from infected tomato in the Netherlands (Mes et al. 1999; Rep et al. 2005), their *SIX4* gene homologs are remarkably conserved and share more than 99% nucleotide and amino acid identity whereas other shared *SIX* genes have much weaker homologies. Furthermore, we show that full Fo5176 virulence on *Arabidopsis* requires the *SIX4* gene, and the attenuated virulence in Fo5176-*SIX4* knockouts can be complemented by the *SIX4/Avr1* gene of *F. oxysporum* f. sp. *lycopersici*. The *SIX4* genes of *F. oxysporum* appear to contribute quantitatively to overall virulence on *Arabidopsis* but act qualitatively as either an Avr gene or as a suppressor of other race-specific resistance in tomato. The high level of sequence conservation in *SIX4* genes between strains infecting these diverse hosts suggests that functions in the two disparate hosts may be mechanistically related and that the genes probably share a recent common origin.

RESULTS

A highly conserved *SIX4* homolog in *Arabidopsis*-infecting strain Fo5176.

To determine if *SIX* genes are present in the *Arabidopsis*-infecting *F. oxysporum* isolate Fo5176, we queried a new 454 shotgun genome sequence assembly (8× coverage, assembled into 7,858 contigs with a total genome size of 55 Mb) of Fo5176 with each of the available *Fol-SIX* gene sequences and identified putative homologs for *SIX1*, *SIX4*, *SIX8*, and *SIX9*

Table 1. Percentage identity and similarity between *Fusarium oxysporum* f. sp. *lycopersici* secreted-in-xylem (SIX) and Fo5176-SIX homologues^a

Fo5176-SIX	GenBank accession	Amino acid identity (%)	Amino acid similarity (%)
SIX1	HQ260601	74	80
SIX4	HQ260602	99.2	99.2
SIX4b ^b	HQ260605	81	89
SIX8	HQ260604	90	94
SIX9	HQ260603	53	66
SIX9b	HQ260606	53	65

^a TBLASTN was used to identify *Fol-SIX* homologues in the Fo5176 genome.

^b Available Fo5176-*SIX4b* sequence does not contain the N-terminal signal peptide; 5' end of this sequence lies at the 5' end of its contig. No overlapping contigs have been identified.

(Table 1). Active genome sequencing of multiple *F. oxysporum* strains is in progress in several laboratories (L.-J. Ma, *personal communication*) and this will probably lead to many variants of *SIX* genes being identified. Therefore, we propose the following nomenclature to identify *SIX* gene homologs; [Fo] [forma specialis acronym if known] [strain code, if forma specialis is unknown or if there is variation within the forma specialis]-[*SIX* number]. For example, the original *SIX4/Avr1* (Houterman et al. 2007) would be termed *Fol-SIX4* in this nomenclature because no sequence variants are known within *F. oxysporum* f. sp. *lycopersici* (M. Rep, *personal communication*).

Amino acid similarities between Fo5176-SIX1 and Fo5176-SIX8 predicted proteins and their *F. oxysporum* f. sp. *lycopersici* homologs were 80 and 94%, respectively, and two Fo5176-SIX9 proteins shared 66% (SIX9) and 65% (SIX9b) similarity with Fol-SIX9. Fo5176-SIX4 was found on a 2,339-bp contig in the Fo5176 genome and this aligned completely at 99% nucleotide identity to the *SIX4* region in *Fol-SIX4*. (Supplementary Fig. S1). Additional analysis identified another *SIX4* homolog encoded on a distinct contig with 89% amino acid similarity with Fol-SIX4 (Table 1; Supplementary Fig. S2). Due to the higher level of conservation in the first *SIX4* homolog, we herein call this Fo5176-SIX4 and call the second putative paralog Fo5176-SIX4b. From cDNA sequencing, the deduced Fo5176-SIX4 coding sequence revealed >99% amino acid identity with Fol-SIX4 and conservation of its single intron site and putative secretion signal sequence. Two single-nucleotide changes in exon two result in two amino acid differences in Fo5176-SIX4 compared with Fol-SIX4; an alanine (A) to glutamic acid (E) and glutamic acid (E) to glycine (G). The Fo5176-SIX4 and Fo5176-SIX4b open reading frames (ORF) share 77.2% nucleotide identity and, based on splice site prediction, Fo5176-SIX4b also contains the conserved *SIX4* intron. However, Fo5176-SIX4b appears to lack the N-terminal signal peptide but a possible start codon of Fo5176-SIX4b lies at the initial 5'

end of its contig. Because an overlapping contig has not been identified, it is unclear whether a signal sequence may exist in SIX4b.

The remarkable sequence conservation between Fo5176-SIX4 and Fol-SIX4 when compared with the sequence homologies in other *SIX* gene orthologs suggested that this gene may have an important function in *F. oxysporum* pathogenesis and, therefore, the role of *SIX4* in the infection of *Arabidopsis* was studied further.

Fo5176-SIX4 is highly expressed during *Arabidopsis* infection.

SIX proteins were originally identified from the xylem sap proteome of *F. oxysporum*-infected tomato plants (Houterman et al. 2007; Rep et al. 2004). To determine whether Fo5176-SIX4 homologs are expressed during infection of *Arabidopsis*, we quantified its mRNA expression (relative to that of fungal β -tubulin) in infected plants and axenic cultures using quantitative real-time PCR. No β -tubulin expression could be detected in leaves, indicating that *Fusarium* sp. growth had not progressed into these tissues at time of sampling. However, both β -tubulin and Fo5176-SIX4 expression were detected in root tissues where Fo5176-SIX4 was expressed at similar levels from 2, 4, and 7 days postinoculation (Fig. 1). Fo5176-SIX4 expression was also detected in *Fusarium* spp. growing in potato dextrose broth (PDB); however, expression levels were 18- to 70-fold less than those measured in planta (Fig. 1), suggesting that its expression is highly responsive to the infection process. Compared with Fo5176-SIX4, the expression of Fo5176-SIX4b was 50- and >100-fold less in culture and in planta, respectively (data not shown), suggesting that Fo5176-SIX4b has little or no functional role in fungal pathogenesis, and this gene was not studied further.

Fo5176 mutants lacking Fo5176-SIX4 have reduced virulence and exhibit reduced growth within host tissue.

To examine the role of Fo5176-SIX4 in infection of *Arabidopsis*, we generated a Fo5176-SIX4 gene knockout construct by replacing 363 bp of Fo5176-SIX4 genomic sequence with the neomycin antibiotic resistance cassette (Fig. 2A). Introduction of this construct into Fo5176 through *Agrobacterium* sp.-mediated transformation generated two independent gene knockout mutants named Δ six4-1 and Δ six4-2 (Supplementary Fig. S3). Compared with *Arabidopsis* plants inoculated with Fo5176, Δ six4-1- and Δ six4-2-inoculated plants showed significantly reduced disease symptom development, with 71 and 42% less chlorosis at 7 days postinoculation and 66 and 28% less necrosis at 10 days postinoculation, respectively (Fig. 2B and C). The survival rates of Δ six4-inoculated plants were also significantly higher than Fo5176-inoculated plants, with 16 and 7 of 60 plants surviving (Δ six4-1 and Δ six4-2, respectively) compared with only 1 of 60 plants for those inoculated with Fo5176 (Fig. 2D). Therefore, in contrast to Fol-SIX4, which is not required for full virulence on tomato (Houterman et al. 2008), our results demonstrated that Fo5176-SIX4 is required for full virulence on *Arabidopsis*. Growth rates and sporulation did not differ between Fo5176 and Δ six4 mutants on agar plates or in broth (data not shown), suggesting that reduced virulence in Δ six4 was not due to slower fungal growth or development.

To determine whether fungal growth was reduced in plants inoculated with a Δ six4 mutant, we quantified fungal biomass in the leaf tissue of plants inoculated with Fo5176 and the Δ six4-1 mutant in a separate experiment (Fig. 3). The reduced disease symptom development observed on Δ six4-1-inoculated plants (Fig. 2E) was associated with a significant reduction in fungal biomass, over 10-fold less than those inoculated with Fo5176 (Fig. 3).

Fig. 1. Fo5176-SIX4 is highly expressed during infection of *Arabidopsis* roots. Fo5176-SIX4 expression was quantified in Fo5176 grown in potato dextrose broth and from infected *Arabidopsis* roots 2, 4, and 7 days postinoculation (dpi). Gene expression levels are relative to the internal control β -tubulin gene. Averages of three (petri dish, in planta) or four (shaking flask) biological replicates are shown with standard errors of the mean. Fo5176-SIX4 expression could not be detected in leaf tissue of infected plants at the time points sampled.

Complementation of $\Delta six4$ mutants with *Fo5176-SIX4* restores full virulence.

To test that the reduced levels of virulence in $\Delta six4$ mutants were due to deletion of *Fo5176-SIX4*, $\Delta six4$ mutants were complemented with a *Fo5176-SIX4* genomic sequence ($\Delta six4$ + *Fo5176-SIX4*) (Fig. 4). Following plant inoculation, both *Fo5176-SIX4*-complemented mutants had a restored full disease phenotype that was not significantly different in disease progression from inoculations with the wild-type fully virulent Fo5176 strain (Fig. 4B and C). Disease development with the complemented mutants and the Fo5176 strain were also both

significantly higher than that observed with the two $\Delta six4$ knock-out mutants (Fig. 4B).

Fol-SIX4 complements reduced virulence of $\Delta six4$ mutants on *Arabidopsis*.

As stated above, the Fol-SIX4 and Fo5176-SIX4 proteins differ by only 2 amino acids. Because Fol-SIX4 has been reported not to be necessary for basal virulence on tomato, it was of interest to determine whether these amino acid changes played a role in virulence functions in *Arabidopsis* when compared with Fo5176-SIX4. When we introduced the *Fol-SIX4*

Fig. 2. Fo5176 mutants lacking Fo5176-SIX4 have reduced virulence. **A**, The $\Delta six4$ mutants were created using *Agrobacterium* sp.-mediated targeted deletion of the *Fo5176-SIX4* gene. The location of the two secreted-in-xylem (SIX)4 exons (black boxes) and single intron (white box) are noted. Binding sites for primers used to confirm mutants are noted with an asterisk. **B** to **E**, *Arabidopsis* plants were inoculated with Fo5176 or two independent $\Delta six4$ mutants ($\Delta six4-1$ and $\Delta six4-2$) and disease symptom development assessed. **B**, Chlorotic leaves/plant 7 days postinoculation; **C**, necrotic leaves/plant 10 days postinoculation; and **D**, survival at 21 days postinoculation. The average of six replicates consisting of 10 plants each is shown with standard errors of the mean. Asterisks indicate values that are significantly different from Fo5176 (** = $P < 0.01$ and * = $P < 0.05$, Student's *t* test). **E**, Disease phenotypes of plants 14 days postinoculation. Representative plants are shown. Similar results were obtained in independent experiments.

gene into a $\Delta six4$ mutant, virulence was restored to the $\Delta six4$ mutants (Fig. 5A and B), suggesting that the 2-amino-acid difference between Fo5176-SIX4 and Fol-SIX4 is not specifically required for Fo5176-SIX4 virulence within the *Arabidopsis*-*F. oxysporum* pathosystem. To test whether *Fol-SIX4* might have a nonspecific effect on virulence, we also introduced *Fol-SIX4* into wild-type Fo5176 but found no difference in disease symptom development between the two Fo5176 strains with and without *Fol-SIX4* (Fig. 5C and D). These experiments confirm that *Fol-SIX4* confers the same function as Fo5176-SIX4 in disease symptom development on *Arabidopsis*.

Transgenic *Arabidopsis* plants expressing Fo5176-SIX4 have increased susceptibility.

Because plants inoculated with $\Delta six4$ had reduced disease symptoms, we were interested to determine whether expression of Fo5176-SIX4 in planta would cause increased susceptibility to Fo5176. Therefore, we generated *Arabidopsis* plants expressing the Fo5176-SIX4 coding sequence minus its predicted secretion signal under the control of the strong constitutive *Cauliflower mosaic virus* 35S promoter (35S::Fo5176-SIX4). From independent T2 plant lines, we selected two homozygous lines with the highest Fo5176-SIX4 transcript expression for further analysis in T3 generation (Fig. 6A). Under normal growing conditions, these lines showed no obvious phenotypic differences from wild-type plants (data not shown). Upon inoculation with Fo5176, significantly more 35S::Fo5176-SIX4 plants developed disease symptoms than inoculated wild-type plants (Fig. 6B), suggesting that Fo5176-SIX4 promotes disease development, possibly by acting within host cells.

Host jasmonate-mediated gene expression is reduced in $\Delta six4$ -inoculated plants.

We have previously shown that host jasmonate (JA)-mediated gene expression is induced following *F. oxysporum* infection and that JA signaling promotes infection. For example, the

Fig. 3. Fungal growth is reduced in $\Delta six4$ -inoculated plants. Significantly less fungal biomass was detected in plants inoculated with $\Delta six4-1$ compared with those inoculated with Fo5176. Values are relative to *Arabidopsis* DNA by assaying fungal glyceraldehyde 3-phosphate dehydrogenase (GPD; FOXG_08006) and *Arabidopsis* iASK (At5g26751) sequences from the leaves of inoculated plants by quantitative real-time polymerase chain reaction. The average of three independent replicates consisting of pools of six plants each is shown with standard errors of the mean. Asterisks indicate values that are significantly different (* = $P < 0.05$, Student's t test) from Fo5176.

Arabidopsis mutants *coil* and *pft1* both exhibit a high level of resistance to *F. oxysporum* and have reduced or almost absent activation of JA responses (Kidd et al. 2009; Thatcher et al. 2009). To determine whether the reduced disease symptom development on $\Delta six4$ -inoculated plants was associated with altered JA-mediated gene expression, we quantified the expression of JA biosynthesis (*LOX3* and *OPR3*), JA signaling (*ERF1*), and JA defense genes (*PDF1.2*, *Thi2.1*, and *PR4*) in Fo5176- and $\Delta six4$ -inoculated plants (Fig. 7A to F). Compared with Fo5176 or ectopic control (contains both wild-type Fo5176-SIX4 and knockout construct) *F. oxysporum*-inoculated plants, the expression of all JA-response genes tested showed reduced levels of expression in the $\Delta six4$ -inoculated plants, with reductions ranging from 1.5-fold (*LOX3*) to 4.5-fold (*PDF1.2*).

Because $\Delta six4$ -inoculated plants showed attenuated JA-defense gene expression, we also investigated whether salicylic acid (SA)-responsive defense genes showed any differential expression following inoculation. However, the expression of SA-marker genes *pathogenesis-related (PR)1* and *PR2* did not differ in plants inoculated with $\Delta six4$, suggesting that decreased virulence of $\Delta six4$ was not due to upregulation of SA-mediated defenses (Fig. 7G and H).

DISCUSSION

Since the cloning of the first *SIX* gene from *F. oxysporum* f. sp. *lycopersici* (Rep et al. 2002, 2004), 11 *Fol-SIX* effectors have been identified (Takken and Rep 2010). These small, cysteine-rich proteins are secreted into the xylem of infected tomato plants where roles in virulence or avirulence have been demonstrated for some (Houterman et al. 2007, 2008; Rep et al. 2004). Apart from a *SIX1*-related sequence in *F. oxysporum* f. sp. *dianthi* (Rep et al. 2004), homologs of *SIX1* to *SIX5* have only been reported for *F. oxysporum* f. sp. *lycopersici* isolates (Lievens et al. 2009). Here, however, we identified homologs of *SIX1* and *SIX4*, along with *SIX8* and *SIX9* homologs, in an *Arabidopsis*-infecting *F. oxysporum* isolate. Although the Fo5176-SIX9 homologs shared only 53% amino acid identity with that of *F. oxysporum* f. sp. *lycopersici*, Fo5176-SIX1 and Fo5176-SIX8 shared much higher identity with their *F. oxysporum* f. sp. *lycopersici* homologs (74 and 90%, respectively). *SIX6* and *SIX7* homologs from *F. oxysporum* f. sp. *lilii*, *melonis*, and *radicis-cucumerinum* also share similar levels of amino acid identity with their *F. oxysporum* f. sp. *lycopersici* counterparts (Lievens et al. 2009). Strikingly, however, one of the Fo5176-SIX4 homologs we identified differed by only two nucleotides from *Fol-SIX4* that result in 2 amino acid changes out of 242 (0.8% at the amino acid level). The overall nucleotide identity with *Fol-SIX4* across the entire 2,339-bp region of the Fo5176-SIX4 genome sequence contig was 99%. Such a high level of *SIX* gene conservation has previously been observed only within the same formae speciales among strains of similar host range (Houterman et al. 2009; Lievens et al. 2009; Rep et al. 2004). The presence of these two highly conserved effector genes in strains that are pathogenic on diverse hosts suggests that they either have a very recent common origin or have an evolutionarily conserved function that constrains gene diversification. The latter explanation seems less likely because the strong conservation of these genes extended outside of the coding region.

To dissect the function of Fo5176-SIX4 in the *F. oxysporum*-*Arabidopsis* interaction, we generated two independent *F. oxysporum* strains in which Fo5176-SIX4 had been deleted ($\Delta six4$). Both $\Delta six4$ strains had significantly reduced virulence on *Arabidopsis*, and complementation of these mutants by reintroducing Fo5176-SIX4 restored the wild-type disease phenotype. Furthermore, we generated *Arabidopsis* plants express-

ing *Fo5176-SIX4* and these plants exhibited increased disease symptom development upon *F. oxysporum* infection (Fig. 6). Fol-SIX4, however, is not required for full virulence on tomato because its removal has no impact on the disease phenotype of susceptible plants that contain no *I* genes (Houterman et al. 2008). In *F. oxysporum* f. sp. *lycopersici*-tomato interactions, Fol-SIX4 acts as an Avr protein where it triggers resistance in *I*- or *I-1*-containing tomato and as a suppressor of resistance in *I-2* or *I-3* lines (Houterman et al. 2008). How Fol-SIX4 suppresses resistance is not known; however, it is suggested that it may interfere with either uptake from the apoplast of Fol-SIX1 and Fol-SIX3 or *I-2* and *I-3* functions, or signal transduction downstream of these R proteins (Houterman et al. 2008). In addition, all race 1 *F. oxysporum* f. sp. *lycopersici* strains con-

tain *SIX4* along with the virulence effectors *SIX1* and *SIX3* (Houterman et al. 2008; Lievens et al. 2009; Rep et al. 2005; Takken and Rep 2010). It has been proposed that Fol-SIX4 has probably been acquired and retained because of its ability to suppress *I-2* and *I-3* resistances and that the *I* and *I-1* resistance genes evolved later to recognize Fol-SIX4 as an Avr protein (Houterman et al. 2008). Major gene resistance to specific races of *F. oxysporum* is not known in *Arabidopsis*, where only polygenic resistance has been described (Diener and Ausubel 2005). Therefore, it is unlikely that the *SIX4* virulence function demonstrated here works by suppression of a specific resistance gene. However, it is still possible that it may act on processes common to both tomato and *Arabidopsis* such as mechanisms of defense-related signal transduction.

Fig. 4. *Fo5176-SIX4* complementation restores full virulence in $\Delta six4$ mutants. **A**, The $\Delta six4$ mutants were complemented ($\Delta six4$ + *Fo5176-SIX4*) by transformation of $\Delta six4$ with -970 to 1,142 bp of the *Fo5176-SIX4* gene. The location of the two secreted-in-xylem (SIX)4 exons (black boxes) and single intron (white box) are noted. **B**, Diseased leaves/plant 7 days postinoculation. The average of six replicates consisting of 10 plants each is shown with standard error. Letters indicate values that are significantly different from each other ($P < 0.05$, all pairs Student's *t* test). **C**, Disease phenotypes of plants inoculated 7 days postinoculation. Representative plants are shown. Similar results were obtained in independent experiments.

The requirement of Fo5176-SIX4 for full Fo5176 virulence on *Arabidopsis* has led us to examine its mode of action in further detail. First, although *Fo5176-SIX4* expression was detectable in culture, its expression increased greatly upon infection of *Arabidopsis* roots (Fig. 1). *Fo5176-SIX4* expression in infected roots was up to 70 times higher than in culture, suggesting that it is highly responsive to infection cues. A similar expression pattern occurs for *Fol-SIX1* during infection of tomato (Rep et al. 2004; van der Does et al. 2008). *Fol-SIX1* expression requires host cells and is highly expressed in root tissues. The lack of or very low levels of *Fol-SIX1* and *Fo5176-SIX4* expression in the absence of a host suggest that *SIX* effector genes are induced specifically during host colonization. It has been suggested by van der Does and associates (2008) that this ability may be used by *F. oxysporum* to switch between its biotrophic invading phase and its necrotrophic phase, where *SIX* effectors may not be necessary.

In tomato xylem sap, several PR proteins have been detected specifically upon *F. oxysporum* infection. These include members upregulated through SA- and JA-signaling pathways such as PR1, glucanase (PR2), chitinase (PR3 and PR4), PR5, and several peroxidases (Houterman et al. 2007; Rep et al. 2002). In the *Arabidopsis*–*Fusarium* pathosystem, *PR* genes regulated

through the SA pathway are either suppressed or nonresponsive to infection (Edgar et al. 2006; Kidd et al. 2011), suggesting that defense responses mediated by SA are not activated during infection. Indeed, genetic studies and hormone treatments indicate only a minor effect of SA on Fusarium wilt disease outcome in *Arabidopsis* (Diener and Ausubel 2005; Edgar et al. 2006; Thatcher et al. 2009). In contrast, we have found that the JA pathway plays a major role in disease development in *Arabidopsis*. For example, the high level of *F. oxysporum* resistance in the *jin1/myc2* mutant is likely mediated, at least in part, by its specific upregulation of JA-mediated defenses (Anderson et al. 2004), while resistance in *coil* and *pft1* mutants is likely mediated by their overall reduction in nondefensive JA responses such as JA-induced senescence (Kidd et al. 2009; Thatcher et al. 2009). A common attribute of these plant mutants is their reduced sensitivity to JA; therefore, we have proposed that *F. oxysporum* hijacks aspects of the JA-signaling pathway to promote wilt-disease symptoms that eventuate in plant death. Interestingly, the reduced disease symptom development in plants inoculated with Fo5176 lacking *SIX4* ($\Delta six4$) was associated with a significant reduction in the expression of JA-defense, -biosynthesis, and -signaling genes, whereas there was no difference in SA-marker gene expression (Fig. 7). This sug-

Fig. 5. *Fol-SIX4* complements $\Delta six4$ virulence but does not affect virulence or avirulence in wild-type *Fusarium oxysporum* Fo5176. **A** and **B**, Mutant $\Delta six4$ was complemented ($\Delta six4$ + *Fol-SIX4*) by transformation of $\Delta six4$ with -708 to 1,142 bp of the *Fol-SIX4* gene. **C** and **D**, Fo5176 transformed with -708 to 1,142 bp of the *Fol-SIX4* gene (Fo5176 + *Fol-SIX4*). **A** and **C**, Diseased leaves/plant 14 days postinoculation. **B** and **D**, Survival at 21 days postinoculation. The $\Delta six4$ + *Fol-SIX4* and Fo5176 + *Fol-SIX4* lines a and b represent independent transformants. The average of four replicates consisting of seven plants each is shown with standard errors of the mean. Letters indicate values that are significantly different from each other ($P < 0.05$, all pairs Student's *t* test). Similar results were obtained in independent experiments.

gested that the virulence activity of Fo5176-SIX4 may function through activation of host JA responses. Bacterial effectors unrelated to *SIX4* proteins that promote jasmonate signaling to induce host susceptibility have been identified (Cui et al. 2010). To determine whether Fo5176-SIX4 itself could induce or prime host JA responses in the absence of other *Fusarium* sp. elicitors, we also examined gene expression in *35S::Fo5176-SIX4* transgenic *Arabidopsis* plants. With the exception of the JA-repressor gene *JAZ10*, we were unable to observe consistent and uniform upregulation of JA-responsive genes in both of the *35S::Fo5176-SIX4* lines (data not shown). This suggests that Fo5176-SIX4 is unable to induce host JA responses on its own.

The finding that Fo5176-SIX4 did not activate host JA responses when transgenically expressed from the host genome, at least on its own, but did cause increased disease symptom development upon infection suggests that Fo5176-SIX4 either acts through other pathways to enhance virulence or requires other *Fusarium* effectors or elicitors to participate in the hijacking of host JA signaling. If Fo5176-SIX4 acts via other virulence mechanisms, then the reduced JA-associated gene expression following infection by $\Delta six4$ was most probably a consequence of much less fungal biomass being produced by $\Delta six4$ than the Fo5176 wild-type and, therefore, reduced colonization and less ability to stimulate the JA pathway (Fig. 3). The SIX4 protein of *F. oxysporum* f. sp. *lycopersici* is known to be a secreted protein during colonization of tomato (Houterman et al. 2007), and complete conservation of the signal peptide between Fol-SIX4 and Fo5176-SIX4 indicates that Fo5176-SIX4 is also secreted. Our plant transformation construct was designed to express the Fo5176-SIX4 protein without its signal peptide directly in the plant cytoplasm. The enhanced susceptibility of plants expressing the putative mature Fo5176-SIX4 protein is consistent with it having a role in virulence and also that its site of action is within the host cell cytoplasm. Fol-SIX3 is also recognized intracellularly, implying uptake of SIX proteins by host cells (Houterman et al. 2009). Our experiments, however, do not rule out the possibility that Fo5176-SIX4 may also have a virulence function in the apoplast.

Several copies of *SIX* genes have previously been observed in *F. oxysporum* f. sp. *lycopersici* isolates, with several containing two *SIX1* genes (Rep et al. 2004) and several copies of

SIX8 existing in Fol4287 (M. Rep and L. J. Ma, *unpublished*). We also identified a second *Fo5176-SIX4* gene and two *Fo5176-SIX9* homologs. Although we found that Fol-SIX4 fully complemented the virulence function of Fo5176-SIX4 (Fig. 5), the 2-amino-acid changes between SIX4 from *F. oxysporum* f. sp. *lycopersici* and Fo5176 may affect its recognition within the host. Indeed, a single nucleotide change in Fol-SIX3 affects its recognition by the R protein I-2 but not its virulence on tomato plants lacking I-2 (Houterman et al. 2009). Our finding that addition of Fol-SIX4 to wild-type Fo5176 did not affect disease symptom progression (Fig. 5C and D) demonstrates that the 2-amino-acid differences between Fo5176-SIX4 and Fol-SIX4 are functionally benign and do not confer either added virulence or avirulence functions to Fol-SIX4 on *Arabidopsis*.

With the exception of *Fol-SIX8*, which is present on multiple chromosomes, all *Fol-SIX* genes in the sequenced race 2 strain Fol4287 are located on chromosome-14 that is supernumerary within *F. oxysporum* f. sp. *lycopersici* (Ma et al. 2010). Furthermore, Ma and associates (2010) demonstrated that chromosome 14 of *F. oxysporum* f. sp. *lycopersici* could transfer vegetatively to otherwise genetically isolated strains and confer virulence on tomato, suggesting that effector genes may evolve independently of the basal genome on supernumerary mobile chromosomes. *Fol-SIX4* is absent from the sequenced reference strain Fol4287 and all other race 2 strains; however, recent analysis of diverse *F. oxysporum* f. sp. *lycopersici* strains has demonstrated that *Fol-SIX4* is not located on chromosome 14 but on another putative supernumerary chromosome (M. Rep, *personal communication*). Thus, the acquisition of *SIX4* by strains of *F. oxysporum* f. sp. *lycopersici* and Fo5176 was also probably a result of a horizontal chromosome transfer event. It is likely that *SIX* genes located on distinct supernumerary chromosomes may be mobilized and evolve independently, and that separate chromosomal origins, compared with that observed between the other *SIX* genes, may explain the substantial sequence conservation of *Fo5176-SIX4* with *Fol-SIX4*. The high level of *Fol-SIX4* and *Fo5176-SIX4* gene conservation suggests that *SIX4* transfer occurred recently and was probably from a related source. Because the *SIX4* gene appears to play a critical role in *F. oxysporum* virulence on multiple

Fig. 6. *Arabidopsis* plants expressing *Fo5176-SIX4* are more susceptible to *Fusarium* disease symptom development. Wild-type (WT) and *Fo5176-SIX4*-expressing (*35S::Fo5176-SIX4*) plants were inoculated with Fo5176. **A**, Prior to fungal inoculation, *Fo5176-SIX4* expression was measured in planta. **B**, Diseased plants at 21 days postinoculation. Averages of three replicates (gene expression) or six replicates (disease phenotypes) consisting of pools of 10 plants are shown with standard errors of the mean. Asterisks indicate values that are significantly different (* = $P < 0.05$, Student's *t* test) from the WT. Similar results were obtained in independent experiments.

hosts, its molecular mode of action warrants further investigation.

MATERIALS AND METHODS

SIX gene identification and sequence analysis.

The *F. oxysporum* f. sp. *lycopersici* *SIX* protein sequences were obtained from GenBank accessions ACY39281 to ACY39287 and ACN69118, and from the Broad *Fusarium* website, FOXG_14223 and FOXG_17457. Fo5176 genomic sequence was previously obtained using Illumina sequencing (Ma et al. 2010) but additional sequencing was performed here using 454 technology. The 454 sequencing was performed using GS-FLX Titanium chemistry on a shotgun library sequenced across two plates. Sequencing and de novo assembly were performed by the Australian Genome Research Facility. The genome assembly consisted of 7,858 contigs greater than 199 bp in size, with an average coverage eightfold and an N50 of 60 kb. The genome was annotated for gene content using FGENESH (Salamov and Solovyev 2000) as part of the Mol-Quest2 package. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank databases under the accession AFQF00000000. The version described in this article is the first version, AFQF01000000.

In-house TBLASTN (Altschul et al. 1997; Altschul et al. 2005) against the consensus contig sequences was used to identify Fo5176-*SIX* homologs in the Fo5176 genome. Contigs containing *SIX* homologs were extracted and, for *SIX8* and

SIX9 homologs, gene structure was predicted using homology to *F. oxysporum* f. sp. *lycopersici* *SIX* proteins with FGENESH+ (Softberry, Mount Kisco, NY, U.S.A.), because these genes had been missed altogether or incorrectly predicted with the initial ab initio prediction. After manual removal of a small number of very short incomplete gene predictions, the final gene number was 17,817. Locus numbers are in the format FOXB_XXXXX. Fo5176-*SIX* gene homologs are available at GenBank (HQ260601 to HQ260606). Amino acid and nucleotide alignments were performed with ClustalW and Align (available on the world wide web).

Quantitative reverse-transcription (Q-RT)-PCR.

For *F. oxysporum* gene expression, Q-RT-PCR experiments were performed from tissue grown in PDB or harvested from infected *Arabidopsis* roots. For stationary cultures, 100 µl of 1×10^6 spores/ml were transferred into a petri dish containing 50 ml of PDB and incubated at room temperature. For the shaking cultures, a flask of PDB was inoculated with an *F. oxysporum* agar plug. After 3 days, the resulting mycelia and spores were harvested through Miracloth (Calbiochem, San Diego, CA, U.S.A.), rinsed with sterile water, and frozen in liquid nitrogen. Three biological replicates were taken for stationary cultures and infected root samples and four for shaking flask samples. RNA extraction, cDNA synthesis, and Q-RT-PCR were conducted as described by McGrath and associates (2005) using an Applied Biosystems 7900HT Fast Real-Time PCR System (Foster City, CA, U.S.A.). Absolute gene expression levels

Fig. 7. Host jasmonate (JA)-mediated gene expression is reduced in $\Delta six4$ -inoculated plants. Expression of **A** and **B**, JA biosynthesis; **C**, the positive JA regulator *ERF1*; and **D** to **F**, JA-mediated defense gene expression is reduced in plants inoculated with $\Delta six4$. **G** and **H**, Reduced activation of JA-mediated gene expression does not correlate with increased salicylic-acid-mediated gene expression. The average of three independent replicates consisting of pools of 10 plants each is shown with standard error. Asterisks indicate values that are significantly different (* = $P < 0.05$, Student's *t* test) from Fo5176. An ectopic strain was used as a *Fusarium* transformation control and contains the wild-type Fo5176-*SIX4* gene along with the knockout construct.

relative to the β -tubulin reference gene were used for each cDNA sample using the equation: relative ratio gene of interest/ β -Tub = (E_{gene}^{-Ct_{gene}})/(E _{β -Tub}^{-Ct _{β -Tub}}). β -Tubulin primers were designed from FOXG_06228 and were Fo β -Tub-F 5'-TGTTT GACCCCAAGAACAT-3' and Fo β -Tub-R 5'-GGTCCTCGAC CTCCTTCATA-3'. Fo5176-SIX4 primers were designed from the *F. oxysporum* f. sp. *lycopersici* sequence AM234064 and were SIX4RT-F 5'-GACGGGCATTATGGACAAAC-3' and SIX4RT-R 5'-CGCTGGAAGGATCAAAGTTC-3'.

For *Arabidopsis* gene expression, Q-RT-PCR experiments were performed on shoot tissue collected before or 4 days after *F. oxysporum* inoculation. Three biological replicates were taken, consisting of tissue pooled from 5 to 10 plants. Plants were cut at the top of the root, just below the crown; therefore, shoot tissue consisted of the hypocotyl and aerial tissue. Absolute gene expression levels relative to the previously validated reference genes β -actin 2, β -actin 7, and β -actin 8 (At1g49240, At3g18780, and At5g09810, respectively) were used for each cDNA using (E_{gene}^{-Ct_{gene}})/(E_{actin}^{-Ct_{actin}}). The gene-specific primer sequences have been previously published (Anderson et al. 2004; Edgar et al. 2006; Thatcher et al. 2009).

Fo5176-SIX4 disruption and complementation.

Approximately 2.2 kb of Fo5176-SIX4 spanning from -730 bp (promoter relative to start codon) to 1,476 bp (ORF and 3' untranslated region [UTR]) was amplified off genomic DNA and cloned into pPZP200 in DY380 *Escherichia coli*. (Fo_SIX4 -730PstI-F 5'-GGTCTGCAGACACACTGGTCGACTTAGA-3' Fo_SIX4 3'UTR KpnI-R 5'-GGCGGTACCATCGAGAAGT GGTGGTCAA-3'). The neomycin cassette for insertion into FoSIX4-pPZP200 was amplified from pAN9.1 using Fo_SIX4 NeoRBM-F 5'-GGGTGACATCATCGGAACATTCAACTTT AGCAGCTCAGACAGTCAAGAAGAACTGGAGGGGTG-3' and Fo_SIX4 NeoRBM-R 5'-CATGTGTTGCCTCCTTGTCATCTACCGCATCAAAAACAGTGTTAGTAACCTGAGGCTA TGGCA-3' and then recombined according to Gardiner and associates (2009). The resulting plasmid, FoSIX4-Neo-pPZP200, was transformed into DH10 *E. coli* to remove any nonrecombined plasmid. FoSIX4-Neo-pPZP200 was then transformed into *Agrobacterium* AGL1 and was used to transform Fo5176 according to Kidd and associates (2011). Transformants were selected on potato dextrose agar (PDA) with G418 (Sigma-Aldrich, St Louis) at 50 mg/liter and confirmed by PCR from individual DNA extractions using SIX4-F (5'-GTTATTGCTTCGGTGGCTGT-3') and SIX4-del-R (5'-CAG ACTCGGTATGACAGAT-3') (for absence of wild-type band) and SIX4-F to Neo-R (5'-ACGTTTGACTTGCAATGTGC-3') (for presence of neomycin cassette). The Δ six4 knockouts were purified via single-spore isolation, confirmed by PCR, and stored dry on filter paper at 4°C.

Complementation of Δ six4 was achieved by transformation with -970 bp (promoter relative to start codon) to 1,142 bp of Fo5176-SIX4 genomic sequence using Fo_SIX4 -970 BamHI-F 5'-CTCGGATCCAAAGAAGTTATTACCGAT-3' and Fo_SIX4 +1,142 MfeI-R 5'-CGGCAATTGCGGTCAAGA AATTAAAGAT-3'. The resulting amplicon was cloned into BamHI/EcoRI-digested pPZPnat1 (Gardiner et al. 2005) to produce FoSIX4-comp-pPZPnat1, moved into *Agrobacterium* AGL1, and transformed into Δ six4 to produce Δ six4 + Fo5176-SIX4. Transformants were selected on PDA with nourseothricin (Werner BioAgents, Jena, Germany) at 50 mg/liter and confirmed by PCR from individual DNA extractions using SIX4-F and SIX4 del-R (for presence of the wild-type band). Two complements for each Δ six4 strain were single spored, confirmed by PCR, and stored on filter paper.

Complementation of Δ six4 or Fo5176 with Fol-SIX4 was achieved by amplification of -708 to 1,142 of Fol-SIX4 from

the vector SIX4 pRW1p (Houterman et al. 2008) using Fol_SIX4 -708 BamHI-F 5'-CGAGGATCCGTTTACTCCGC CTGGCTAC-3' and Fo_SIX4 +1,142 MfeI-R. The amplicon was cloned into pPZPnat1 and moved into *Agrobacterium*. Transformants were selected and confirmed as above.

Plant material and growth conditions.

Unless otherwise specified, all experiments were conducted with the *Arabidopsis thaliana* Columbia-0 (Col-0) ecotype grown in soil under a short-daylight regime (8 h of light and 16 h of darkness) at 21°C as described previously (Campbell et al. 2003; Edgar et al. 2006). To clone the Fo5176-SIX4 coding sequence (CDS) for its expression in *Arabidopsis*, total RNA was isolated from mycelia grown in PDB and used to generate cDNA as described above for Q-RT-PCR. For generation of plants expressing the Fo5176-SIX4 CDS minus the signal peptide (35S::Fo5176-SIX4), the Fo5176-SIX4 CDS was amplified using Fo_SIX4 HindIII-F 5'-TGGCTAAGCTTAACAATGCT TCCAAAGGGGGAG-3' which includes an ATG start codon, and Fo_SIX4 EcoRI-R 5'-CTTAGAATTCCTAAGCTAAGTT AAGTGT-3'. Removal of the signal peptide results in a mature SIX4 protein starting with the amino acids MLPKGEE. The resulting amplicon was cloned into HindIII/EcoRI-digested binary vector pKEN (McGrath et al. 2005) and confirmed by sequencing. 35S::Fo5176-SIX4 pKEN was mobilized into *Agrobacterium* AGL1 and transformed into *A. thaliana* as per McGrath and associates (2005). Transgenic plants were selected based on their resistance to Pestanal (glufosinate-ammonium; Riedel-de Haen, Seelze, Germany) at 10 mg/liter and resulting T3 lines were used in subsequent experiments.

F. oxysporum inoculation.

The *F. oxysporum* isolate used in this study was strain Fo5176 obtained from R. Shivas, Queensland Plant Pathology Herbarium, Queensland Department of Primary Industries and Fisheries, Brisbane, Australia. Inoculations were performed as described by Anderson and associates (2004) and Edgar and associates (2006).

Fungal biomass estimation.

Fungal biomass measurements were performed as described previously (Thatcher et al. 2009) with whole aboveground tissues for DNA extractions collected at 10 days postinoculation.

F. oxysporum growth assay.

Strains Δ six4 and its complement Δ six4 + Fo5176-SIX4 were grown on an appropriate antibiotic selection and then an infected agar plug transferred to media without selection (PDA). The radius was measured daily for 7 days and the growth rate per day (in millimeters) was determined.

ACKNOWLEDGMENTS

L. F. Thatcher is supported by a CSIRO OCE postdoctoral fellowship. We thank R. Shivas for the *F. oxysporum* (strain Fo5176); J. Hane for assisting with Fo5176 genome submission to GenBank; P. Houterman for the SIX4 pRW1p vector; A. Rusu, A. Kettle, and H. Zhao for technical assistance in *F. oxysporum* inoculation experiments; and B. Kidd for critical reading of the manuscript and useful discussions.

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