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The *FTF* gene family regulates virulence and expression of SIX effectors in *Fusarium oxysporum*

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SUMMARY

The *FTF* (*Fusarium transcription factor*) gene family comprises a single copy gene, *FTF2*, which is present in all the filamentous ascomycetes analysed, and several copies of a close relative, *FTF1*, which is exclusive to *Fusarium oxysporum*. An RNA-mediated gene silencing system was developed to target mRNA produced by all the *FTF* genes, and tested in two formae speciales: *F. oxysporum* f. sp. *phaseoli* (whose host is common bean) and *F. oxysporum* f. sp. *lycopersici* (whose host is tomato). Quantification of the mRNA levels showed knockdown of *FTF1* and *FTF2* in randomly isolated transformants of both formae speciales. The attenuation of *FTF* expression resulted in a marked reduction in virulence, a reduced expression of several *SIX* (*Secreted In Xylem*) genes, the best studied family of effectors in *F. oxysporum*, and lower levels of *SGE1* (*Six Gene Expression 1*) mRNA, the presumptive regulator of *SIX* expression. Moreover, the knockdown mutants showed a pattern of colonization of the host plant similar to that displayed by strains devoid of *FTF1* copies (weakly virulent strains). Gene knockout of *FTF2* also resulted in a reduction in virulence, but to a lesser extent. These results demonstrate the role of the *FTF* gene expansion, mostly the *FTF1* paralogues, as a regulator of virulence in *F. oxysporum* and suggest that the control of effector expression is the mechanism involved.

Keywords: effector, *FTF*, Fusarium wilt, genomic expansion, pathogenicity, transcription factor, virulence.

INTRODUCTION

Fusarium oxysporum Schlechtend.:Fr. is an anamorphic species complex (*Fusarium oxysporum* species complex, FOSC) ubiquitous in soils worldwide and able to grow as a saprophyte or by the colonization of plants. The pathogenic strains collectively may infect more than 100 different hosts, many of them important crops (Michielse and Rep, 2009), but the individual isolates are able to infect only one or a few plant species, allowing for classification into host-specific forms, known as formae speciales (Armstrong and Armstrong, 1981). The comparison between the genomes of the different *F. oxysporum* formae speciales sequenced to date and those of the two other species of the *Fusarium* genus, *F. graminearum* and *F. verticillioides*, has revealed a well-conserved core genome in the three species and several lineage-specific (LS) genomic regions in *F. oxysporum* (Ma *et al.*, 2010). LS regions are mostly organized into supernumerary chromosomes, which contain genes that are not required for basic metabolic processes and are absent in closely related species. The LS regions of *F. oxysporum* f. sp. *lycopersici* include four entire chromosomes (chromosomes 3, 6, 14 and 15), parts of chromosome 1 and 2, and some small scaffolds. It has been shown that the transfer of some LS chromosomes between strains of *F. oxysporum* may convert a non-pathogenic strain into a pathogen, thus demonstrating the importance of the LS part of the genome in pathogenicity. These regions show a surprising enrichment of transposable elements and genes predicted to encode secreted proteins, some specifically expressed during plant infection, such as the *SIX* (*Secreted In Xylem*) genes (Houterman *et al.*, 2008; Rep *et al.*, 2004).

Among the gene families expanded in the LS genome of *F. oxysporum*, it is worthwhile to highlight *FTF1* (*Fusarium transcription factor 1*). This gene putatively encodes a $Zn(II)_2Cys_6$ transcription factor and was first described in highly virulent (HV) strains of *F. oxysporum* f. sp. *phaseoli* (Ramos *et al.*, 2007) as part of a SCAR (Sequence Characterized Amplified Region) designed for *in planta* detection (Alves-Santos *et al.*, 2002b). mRNA transcribed from the multiple copies of *FTF1*, or at least some of them, is abundant in plants colonized by *F. oxysporum*, but barely detectable in cultures of the fungus (Ramos *et al.*, 2007). The

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expression level of the gene correlates with the number of copies in strains of *F. oxysporum* f. sp. *phaseoli* and the degree of virulence displayed by these strains when colonizing *Phaseolus vulgaris* L. and *Phaseolus coccineus* L. plants (Alves-Santos *et al.*, 2002b; de Vega-Bartol *et al.*, 2011). Eleven homologues of the gene have been detected in the genome of the tomato pathogen *F. oxysporum* f. sp. *lycopersici* strain 4287 (de Vega-Bartol *et al.*, 2011). Three of these copies (FOXG_16414, FOXG_14257 and FOXG_17458) are located in chromosome 14 close to mini-clusters of *SIX* effector-encoding genes (Schmidt *et al.*, 2013). Recently, it has been shown that *FTF1* is highly expressed in root crown and hypocotyl of common bean plants inoculated with an HV strain of *F. oxysporum* f. sp. *phaseoli* (Niño-Sánchez *et al.*, 2015). Furthermore, up-regulation of *FTF1* correlates with the highest level of expression of the effector-encoding genes *SIX1* and *SIX6*, which takes place when fungal growth is restricted to the vascular system (Niño-Sánchez *et al.*, 2015). Another gene expansion in *F. oxysporum*, *EBR1* (*Enhanced Branching 1*), has been shown to be involved in virulence, similar to its counterpart in *F. graminearum* (Jonkers *et al.*, 2013; Zhao *et al.*, 2011). *EBR1* is located in chromosome 7 of *F. oxysporum* f. sp. *lycopersici*, and thus is part of the core genome, whereas the other EBR paralogues are located in LS chromosomes. Mutants altered in *EBR1* showed reduced growth when grown in culture and reduced virulence against tomato plants (Jonkers *et al.*, 2013).

In this work, we addressed the structure of the *FTF* gene family of *F. oxysporum*, and focused on the functional role of the *FTF1* paralogues by reducing their expression through gene silencing.

RESULTS

FTF2 is a single copy gene with similarity to *FTF1*

Early studies on *FTF1* showed that HV (when inoculated in common bean plants) and supervirulent (SV, when inoculated in runner bean plants) isolates of *F. oxysporum* f. sp. *phaseoli* harboured four and five copies of this gene, respectively (Ramos *et al.*, 2007; de Vega-Bartol *et al.*, 2011). However, a faint hybridization signal could also be detected in digested DNA of these strains, non-pathogenic and weakly virulent (WV, when inoculated in common bean plants) strains (Fig. 1), suggesting that all of these strains harbour a second gene able to cross-hybridize with *FTF1*. We identified two recombinant phages when a genomic library from the WV strain FOP-SP4 was hybridized with a probe containing part of the *FTF1* promoter and the beginning of the open reading frame (ORF). DNA from both phages was subcloned into pBluescript KS+ and the *FTF1* hybridizing region contained in both was sequenced. The coding region of the gene detected in the cloned DNA fragment comprises 3219 bp interrupted by a 49-bp intron located in the same position as that of the intron found in *FTF1*. The predicted polypeptide is 1072 amino acids in length

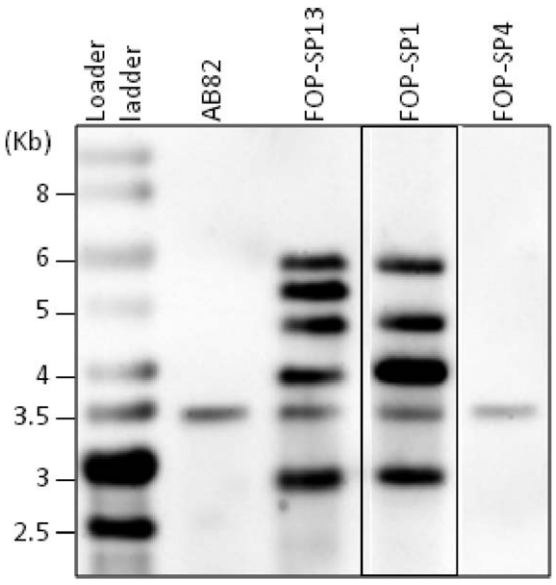

Fig. 1 Southern hybridization of *Hind*III-digested DNA from different strains of *Fusarium oxysporum*. The membrane was hybridized with the probe M18B-M19A which is a segment of the central coding region of the *FTF1* (*Fusarium transcription factor 1*) gene. Size markers are indicated on the left. AB82 is a non-pathogenic strain, FOP-SP13 is a supervirulent (SV) strain, FOP-SP1 is a highly virulent (HV) strain and FOP-SP4 is a weakly virulent (WV) strain.

and contains the $Zn(II)_2Cys_6$ binuclear cluster DNA-binding motif and a 'fungal-specific transcription factor domain' or 'medium homology region' (MHR) (Schjerling and Holmberg, 1996).

Hybridization of several probes derived from the *FTF2* coding region to digested genomic DNA from several *F. oxysporum* strains (non-pathogenic, WV, HV and SV) showed a single hybridizing fragment in non-pathogenic and WV strains, and several fragments corresponding to the *FTF2* gene and the known copies of the *FTF1* gene in the HV and SV strains (data not shown). Sequencing of the DNA insert cloned in one of the recombinant phages revealed an ORF corresponding to a homologue of the *BimB* gene of *Aspergillus nidulans* (May *et al.*, 1992) 5 kb upstream of *FTF2*. Hybridization of the above-described genomic DNA with a probe derived from the *F. oxysporum BimB* homologue revealed a single hybridizing fragment of the same size in all the strains analysed (data not shown), indicating that *FTF2* is a single copy gene with a genomic location different from that of the *FTF1* copies. Further confirmation of the single copy nature of *FTF2* was obtained when promoter and ORF fragments of *FTF2* were PCR amplified from a set of HV, WV and non-pathogenic strains. Sequencing of the amplicons revealed that the fragments were 100% identical.

A BLAST analysis of the 12 available sequenced genomes in FOSC yielded a unique *FTF2* homologue in all isolates (Table 1). All the putative proteins identified as FTF2 transcription factors

Table 1 FTF1 (*Fusarium transcription factor 1*) and *FTF2* (*Fusarium transcription factor 2*) copies in *Fusarium oxysporum* formae speciales.

Formae speciales	*FTF2*	*FTF1a*	*FTF1b*	*FTF1c*	Truncated
Pisi	FOVG_10613				FOVG_18329
Fo 47	FOZG_12687				FOZG_18011 (1007)
Fo human	FOYG_11202				
radicis-lycopersici	FOCG_10914				FOXG_17757 (446)
lycopersici r. 2	FOXG_09390 Chr 9, Sc 11	FOXG_14257 (1070)‡ Chr 14, Sc 22 FOXG_17458 (1079) Chr 14, Sc 51 FOXG_14422 (1072)* Chr. 15, Sc 24 FOXG_15059 (1072)* Chr 1, Sc 27		FOXG_17123 (930) Chr 6, Sc 22 FOXG_16414 (930) Chr 14, Sc 36 FOXG_14000 (930)† Chr 6, Sc 21 FOXG_17084 (930)† Chr 6, Sc 41 FOXG_12539 (930)† Chr 3, Sc 18 FOXG_12589 (930)† Chr 3, Sc 18	
lycopersici r. 3	FOWG_05099	FOWG_17325 (1070) FOWG_18009 (1079)		FOWG_17814 (930) FOWG_17908 (930) FOWG_17740 (930)	
raphani	FOQG_03479	FOQG_15325 (1071)			FOQG_19052 (914)
cubense	FOIG_05174	FOIG_16560 (1046)	FOIG_16484 (971) FOIG_16630 (969)		
vasinfectum	FOTG_03936	FOTG_16755 (1072) FOTG_17879 (1072) FOTG_18080 (1098)	FOTG_17956 (969)		FOTG_18226 FOTG_18225
arabidopsis	FOXB_12381				FOXB_19743 FOXB_12596 FOXB_18246
melonis	FOMG_08221	FOMG_18985 (1075)	FOMG_18692 (969)	FOMG_18999 (930)	FOMG_18691 FOMG_19647
conglutinans	FOPG_02091				FOPG_19962 FOPG_18130 FOPG_19108
phaseoli§	JN167165	phasl14257 phasl17458 phasl15059			

*,†Identical copies.

‡These copies have an exclusive stretch of amino acids between positions 7 and 17.

§Sequenced paralogues, one more *FTF1* paralogue has been detected by Southern hybridization; phasl followed by a locus number indicates the homologous locus in the *F. oxysporum* f. sp. *lycopersici* genome.

showed similarities higher than 99.7% (in some cases they were identical), indicating that *FTF2* is a unique locus in FOSC.

FTF gene expansion

The use of an *FTF2* sequence, or the previously described *FTF1* sequence, in BLAST analysis of the available genomes of FOSC, yields, in addition to the *FTF2* locus, a number of copies that show a variable degree of similarity and that we collectively denominate as *FTF1* (Table 1). The number of *FTF1* copies or paralogues found in each strain is variable, ranging from none in the *pisi*, biocontrol, human, *radicis-lycopersici*, arabidopsis and *conglutinans* isolates, to 10 in the *lycopersici* race 2 strain (4287). However, the number found in strain 4287 should be approached with caution, as loci FOXG_14422 and FOXG_15059, and loci FOXG_14000, FOXG_17084, FOXG_12539 and FOXG_12589, are identical duplicates. It is worthwhile to highlight that all the copies of *FTF1* in *F. oxysporum* f. sp. *lycopersici* 4287 map to chromosomes 3, 6, 14 and 15 and scaffold 27 (chromosome 1), which are LS regions in this strain (Ma *et al.*, 2010), whereas *FTF2* is located in the core genome (chromosome 9). The analysis of homologies, at both the nucleotide and amino acid levels, between *FTF2* and all the *FTF1* loci in the different formae speciales shows that *FTF2* clusters apart from the *FTF1* copies (Figs 2A,C, S1A, see Supporting Information).

The analysis of the proteins putatively encoded by the different *FTF1* loci shows a range of variation in length, in contrast with the homogeneity of *FTF2*, which allows for the classification of three

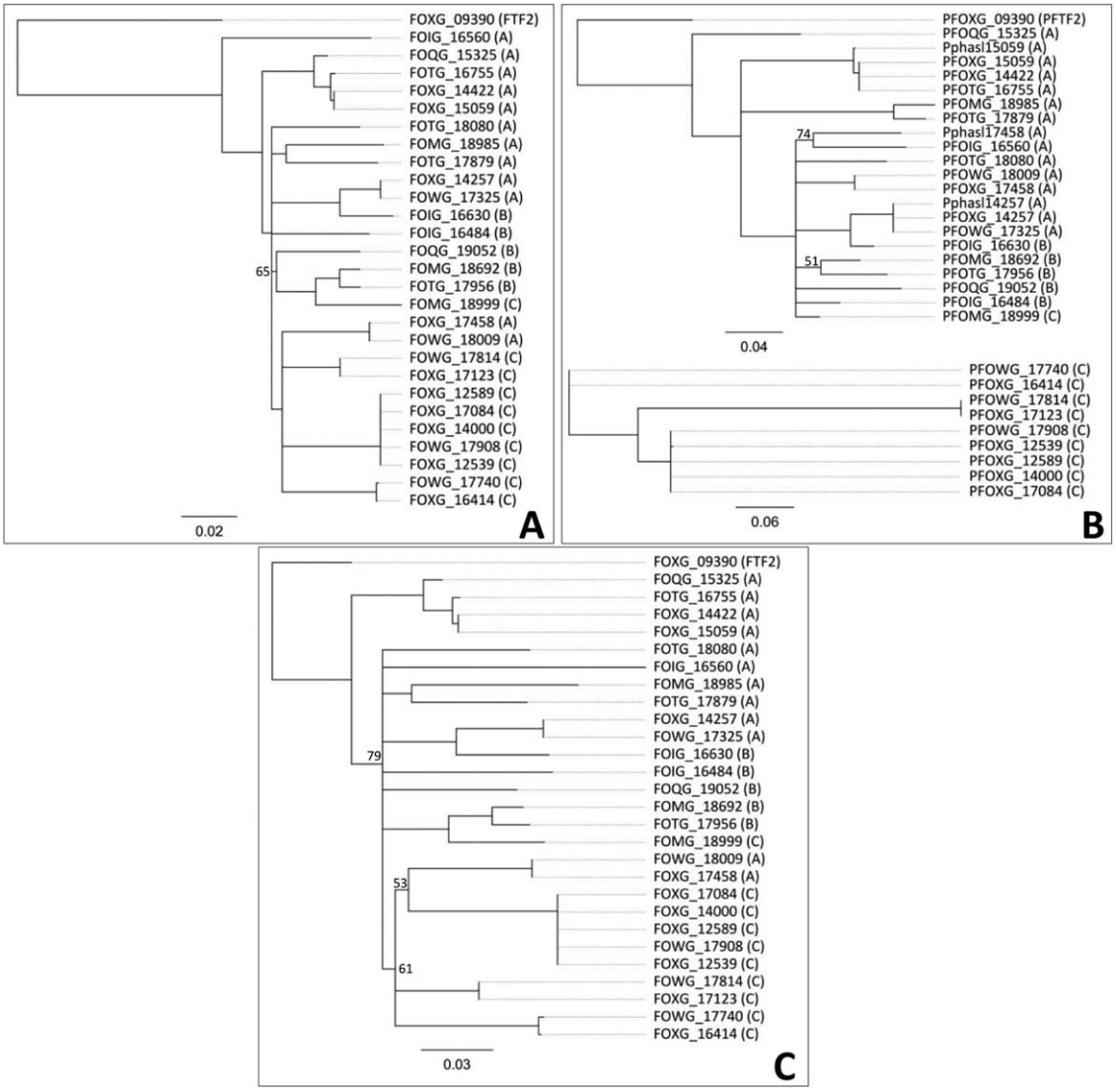

Fig. 2 Similarity between the *FTF2* (*Fusarium transcription factor 2*) and *FTF1* (*Fusarium transcription factor 1*) paralogues, and the deduced proteins, found in the *Fusarium oxysporum* genomes of the different formae speciales. (A) Dendrogram obtained using the alignment of the open reading frames (ORFs). (B) Dendrogram obtained using the alignment of the promoter region (0.5 kb). (C) Dendrogram obtained using the alignment of the deduced proteins. FOXG loci correspond to the f. p. *lycopersici* race 2 isolate (4287), FOIG loci correspond to the f. sp. *cubense* isolate, FOQG loci correspond to the f. sp. *raphani* isolate, FOTG loci correspond to the f. sp. *vasinfectum* isolate, FOMG loci correspond to the f. sp. *melonis* isolate and FOWG loci correspond to the f. sp. *lycopersici* race 3 isolate (see Table 1 for a description of the different loci). The scale indicates the length of branch that represents one substitution per site. Numbers on the branches are bootstrap support values below 80.

basic types (Table 1). The FTF1a type is 1070–1079 amino acids in length in most cases, similar to the FTF2 deduced protein, and can be found in most formae speciales, including three of the *phaseoli* paralogues. The FTF1b type is 969–971 amino acids in length and shows a shorter carboxy-terminus than FTF1a. Paralogues encoding FTF1b are found in the *cubense*, *vasinfectum* and *melonis* isolates. Finally, the FTF1c type of putative protein is 930 amino acids in length, with shorter amino- and carboxy-termini, and is exclusive to f. sp. *lycopersici*.

Apart from these well-defined loci, several truncated copies of *FTF1* are found in most isolates (see Table 1). The alterations shown by these copies range from small internal deletions or a premature stop codon, as in the *raphani* and biocontrol isolates, respectively, to large deletions in the amino- or carboxy-terminus, or the central region of the proteins, which most probably preclude a normal function for these putative proteins.

Former expression analysis has suggested that *FTF2* and *FTF1* respond to different regulatory signals *in planta* (Niño-Sánchez

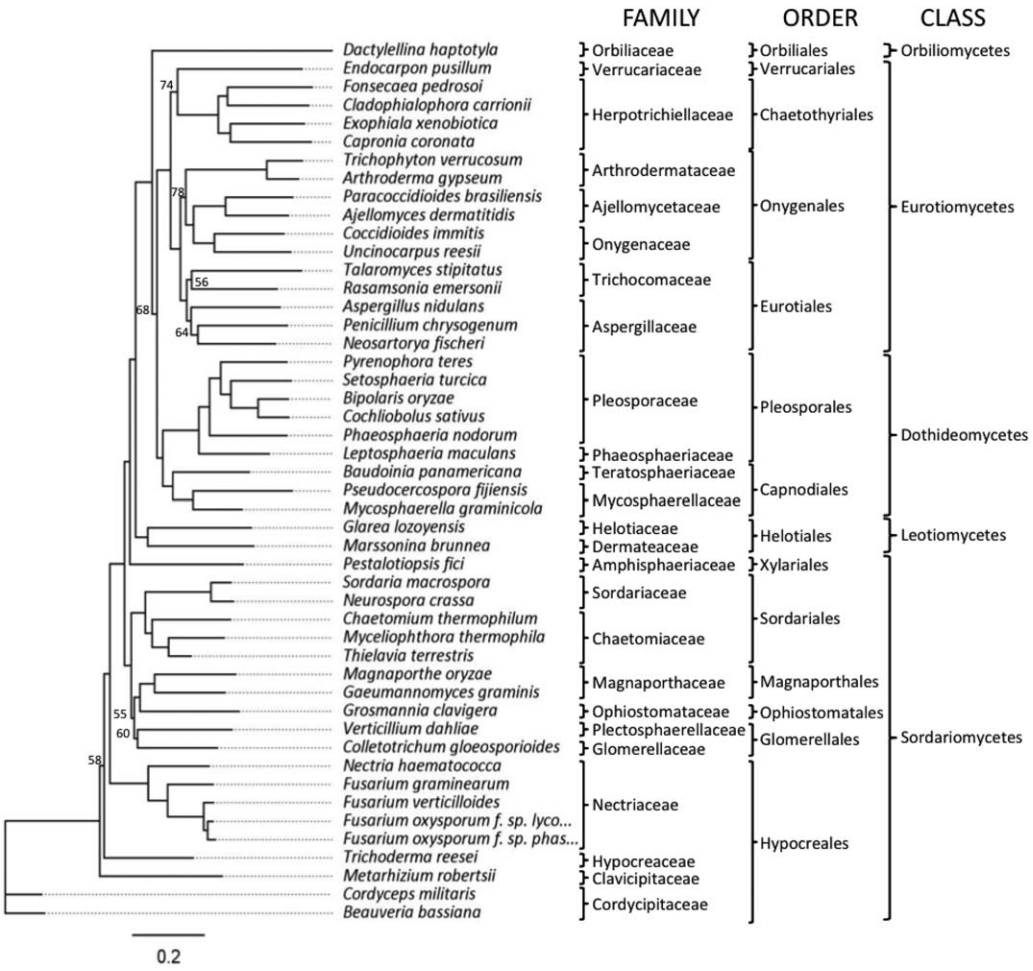

Fig. 3 Genetic similarity between the *FTF2* (*Fusarium transcription factor 2*) homologues in different fungal species. The dendrogram was obtained using the open reading frames (ORFs) of the unique *FTF2* sequences found in the corresponding fungal genomes, except for the *F. oxysporum* f. sp. *lycopersici* and *phaseoli* ORFs, where FOXG_09390 and GenBank JN167165 were used, respectively. The scale indicates the length of branch that represents one substitution per site. On the right, the names of the species, family, order and class are shown. Numbers on the branches are bootstrap support values below 80.

et al., 2015). In addition, *FTF2* shows a steady low level of transcript accumulation during growth in culture, with no significant differences among strains based on virulence (Fig. S2, see Supporting Information). To gain an insight into the regulation of *FTF1* and *FTF2*, we compared a 500-bp promoter stretch immediately upstream of the ATG codon (selected as the codon encoding the methionine of the MSG amino acid sequence). The dendrogram in Fig. 2B shows two distinct groups. The promoters of copies *FTF1a* and *FTF1b* show a clustering pattern similar to that obtained when comparing the ORFs (Fig. 2A). The promoters of the *FTF1c* copies cannot be aligned with the promoters of *FTF1a* and *FTF1b*, except for FOMG_18999, and therefore they cluster in a different group. It is worthwhile to note that the only *FTF1* promoter whose functionality has been demonstrated is *Pphasl14257* in FOP-SP1 (Niño-Sánchez *et al.*, 2015), which is homologous to the promoter found in the FOXG_14257 copy of strain 4287.

To verify whether the *FTF* gene family is present in other fungi, BLAST searches using the *FTF1* and *FTF2* ORFs, or the deduced polypeptides, as query sequences, were performed against other fungal genomes. A single copy gene homologous to *FTF2* was found in the genomes of all the filamentous ascomycetes of the subphyllum Pezizomycotina (Euascomycota) tested, but absent in the genomes of yeasts, basidiomycetes, zygomycetes and chytridiomycetes (Fig. 3). This result indicates that *FTF2* is a single copy gene specific to filamentous fungi, whereas the *FTF1* paralogues and the genomic expansion are exclusive to FOSC.

Analysis of mutants of the *FTF* genes

The relationship between the number of copies of *FTF1* and virulence has been well determined in *F. oxysporum* f. sp. *phaseoli* (Alves-Santos *et al.*, 2002a, b; de Vega-Bartol *et al.*, 2011) and

F. oxysporum f. sp. *dianthi* (Gómez-Lama Cabanás, personal communication, Instituto de Agricultura Sostenible (IAS), Cůrdoba, SPAIN). However, the large number of copies found makes the construction of knockout mutants an insurmountable task. The high similarity between *FTF2* and *FTF1* and among the copies of *FTF1* makes this expansion an attractive target for gene silencing based on RNA interference (RNAi) methodology (Fig. S1B). However, the attenuation of gene expression obtained in this way affects *FTF2* and the copies of *FTF1*. To discriminate the contribution of each gene to the virulence phenotype, we first generated null mutants of *FTF2* in the FOP-SP1 strain. As the genome annotation shows that there is an ORF (FOXG_09391) located contiguous to the 3′ end of the coding region of *FTF2*, a deletion construct was generated using the 5′ end flanking region of *FTF2* (which is specific to *FTF2*) and the 3′ terminus of the *FTF2* coding region (which is highly similar between *FTF2* and *FTF1* copies). This strategy was designed to optimize the probabilities of deleting most of the *FTF*2 coding region without deleting a *FTF1* paralogue or part of the FOXG_09391 coding region, which would result in a double mutation. Several transformants were obtained and subjected to polymerase chain reaction (PCR) and Southern analysis, which verified such a deletion of *FTF2*, but no alteration of the *FTF1* copies (Fig. S3A, see Supporting Information). In addition, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was performed to verify wild-type expression of *FTF1* and the lack of *FTF2* deleted transcript (data not shown). Two transformants were selected for further analyses (SP1Δ*FTF2*.1 and SP1Δ*FTF2*.2). First, it was verified that deletion of *FTF2* did not affect growth and sporulation (Table S1 and Fig. S4, see Supporting Information). Then, they were tested for pathogenicity and virulence in inoculation assays of common bean plants. The results in Fig. 4A–D indicate that both transformants show a slight reduction in virulence as measured by the International Center for Tropical Agriculture (CIAT) scale, plant weight and disease index. The virulence reduction reverted to the wild-type level when the mutation was complemented with the *FTF2* wild-type allele (Figs S3B, 4A–D).

For *FTF* gene silencing, we used a construct based on the strategy developed in *Penicilllium chrysogenum* and *Acremonium chrysogenum* (Ullán *et al.*, 2008) to obtain *FTF*RNAi transformants of the HV *phaseoli* strain FOP-SP1 and the *lycopersici* strain 4287. All the constructs contained a 462-bp *FTF1* fragment to induce gene silencing of *FTF1* and *FTF2* (Fig. S1B), cloned in binary plasmids harbouring either a gene coding for hygromycin resistance (p*FTF*RNAi-Hyg) or phleomycin resistance (p*FTF*RNAi-Phleo). The high similarity between the *FTF1* and *FTF2* ORFs precluded the use of a fragment long enough to induce the specific knockdown of each gene. After transformation of FOP-SP1, the hyg^R transformants SP1*FTF*RNAi.2, SP1*FTF*RNAi.4 and SP1*FTF*RNAi.5, the $phleo^R$ transformant SP1*FTF*RNAi.16 and the hyg^R transformant SP1RNAi, which contains the integrated plasmid backbone without the 462-bp *FTF1* fragment, were selected. Transformants of strain 4287 were obtained in a similar way. Five representative transformants obtained with the silencing construct harbouring the *hph* gene for hygromycin resistance were selected for further analyses, together with a control transformant obtained with the same construct lacking the silencing-inducer *FTF1* fragment. All the selected transformants were analysed by PCR and Southern hybridizations to verify the integration of the silencing construct or the control construct without the *FTF1* fragment (Fig. S3C–G). They were also examined for growth and sporulation in both solid and liquid media. No significant differences could be observed between *FTF*RNAi transformants and their respective wild-type strains (Table S1 and Fig. S4).

Silenced FOP-SP1 transformants were tested for pathogenicty and virulence in common bean plants (*P. vulgaris* L.) (Fig. 4E–H). All transformants were pathogenic, but differed in virulence. Transformants SP1*FTF*RNAi.5 and SP1RNAi showed disease indices similar to those induced by the HV strain FOP-SP1, measured using the CIAT scale, the plant weight of inoculated plants or the disease scale described in Experimental Procedures (Fig. 4F–H, respectively). In addition, the disease progression rate (DPR) was similar. Transformant SP1*FTF*RNAi.4 showed a slightly reduced disease rating, and transformants SP1*FTF*RNAi.2 and SP1*FTF*RNAi.16 showed a significantly reduced disease rating. DPR was also lower for these three transformants (6.25 for SP1*FTF*RNAi.4, 5 for SP1*FTF*RNAi.2 and 4.25 for SP1*FTF*RNAi.16) when compared with FOP-SP1 (DPR = 7). The virulence reduction was precisely correlated with the attenuation of *FTF1* and *FTF2* expression, as measured by RT-qPCR at 7 and 14 days post-inoculation (dpi) (Fig. 4I). The expression data in Fig. 4I show significant differences in the expression of both genes with respect to normal expression in the wild-type strain FOP-SP1. Differences ranged from no reduction in SP1*FTF*RNAi.5 and the control transformant SP1RNAi to more than 50% for *FTF1* in SP1*FTF*RNAi.16.

In a similar way, *FTF*-silenced transformants of the wild-type 4287 were tested in inoculation assays of tomato plants (Fig. 5). The disease indices estimated for plants inoculated with the silenced transformants were, in all cases, lower than those obtained for plants inoculated with the wild-type strain 4287 (Fig. 5C), and the plant survival rate was significantly higher (Fig. 5D). When *FTF1* and *FTF2* expression in the inoculated plants was analysed, the correlation between virulence reduction and attenuated *FTF* expression was not as clear as in common bean inoculations (Fig. 5E). Most transformants showed a reduction in the expression of both genes, but, in all, the expression of each gene was higher than 65% relative to the expression measured in wild-type 4287.

Common bean and tomato plants inoculated with transformants accumulating low levels of *FTF* transcripts showed a clear reduction in Fusarium wilt symptoms. Transformants generated

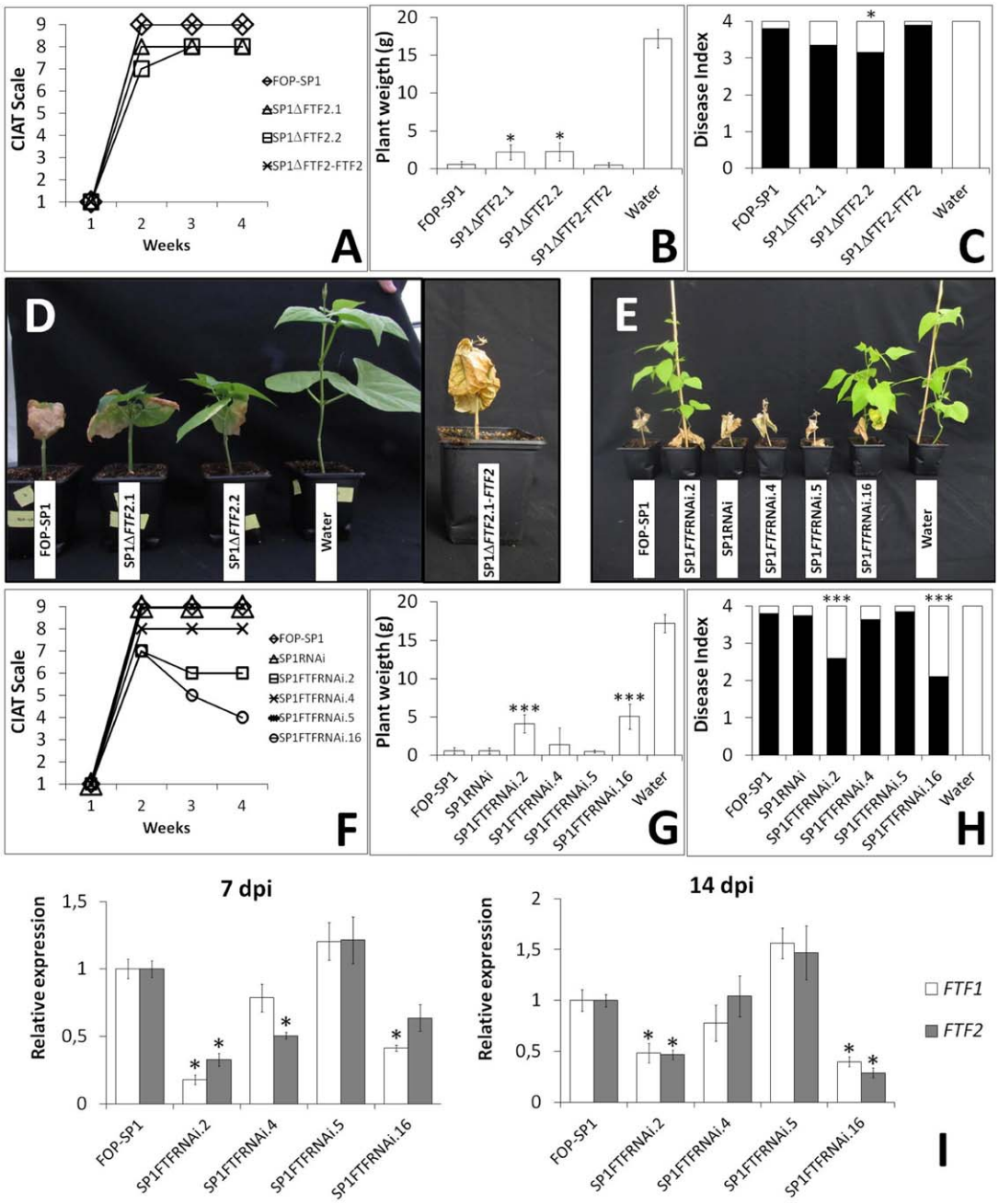

Fig. 4 Fusarium wilt induced in common bean plants by *Fusarium oxysporum* f. sp. *phaseoli* transformants with reduced expression of *FTF* (*Fusarium transcription factor*). Common bean plants 4 weeks after inoculation with *FTF2*$^{-}$ mutants and complemented mutant (A–D) and inoculation with attenuated expression of FTF (E–H). Severity of Fusarium wilt symptoms as measured by: (A, F) the International Center for Tropical Agriculture (CIAT) scale (the disease index is assigned according to the percentage of chlorotic or necrotic leaves); (B, G) the weight of aerial plant parts 4 weeks after inoculation; (C, H) disease index (assigned according to the number of necrotic vascular vessels and whether or not the plant is dead). (I) Relative expression of the *FTF* genes in root crown [7 days post-inoculation (dpi)] and hypocotyl (14 dpi) of colonized plants, as measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). An arbitrary value of 1.0 was used for the transcript level of *FTF* genes in plants infected with FOP-SP1. Error bars in each panel indicate the standard deviations in three independent biological experiments. The differences between each transformant and the wild-type strain in plant weight (B, G), disease index (C, H) and expression of the *FTF* genes (I) were tested using one-way analysis of variance (ANOVA) followed by Dunnett's test, and indicated by $*P < 0.05$ and $***P < 0.001$. FOP-SP1 is a highly virulent (HV) wild-type strain; SP1*FTF*RNAi.2, SP1*FTF*RNAi.4 and SP1*FTF*RNAi.5 are hygromycin-resistant transformants; and SP1*FTF*RNAi.16 is a phleomycin-resistant transformant harbouring an *FTF1* fragment for the induction of gene silencing; SP1RNAi is a hygromycin-resistant strain which lacks the silencing *FTF1* fragment.

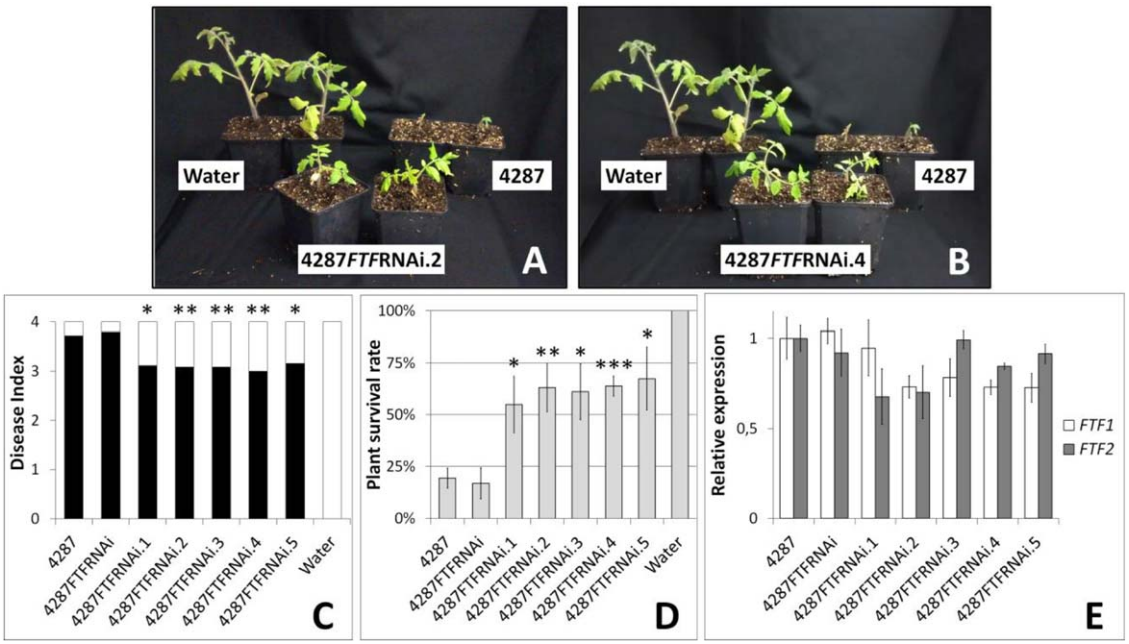

Fig. 5 Fusarium wilt induced in tomato plants by *Fusarium oxysporum* f. sp. *lycopersici* attenuated transformants. (A, B) Tomato plants 3 weeks after inoculation. Severity of Fusarium wilt symptoms 3 weeks after inoculation, as measured by the disease index (C) and plant survival rate (D). (E) Relative expression of the *FTF* (*Fusarium transcription factor*) genes in the root crown of colonized plants at 14 days post-inoculation (dpi), as measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). An arbitrary value of 1.0 was denoted for the transcript level of *FTF* genes in plants infected with the wild-type strain 4287. Error bars indicate the standard deviations obtained in three independent biological experiments. The differences between each transformant and the wild-type strain in the disease index (C) and plant survival rate (D) were tested using one-way analysis of variance (ANOVA) followed by Dunnett's test, and are indicated by $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Transformants 4287*FTF*RNAi.1-5 and 4287*FTF*RNAi were obtained with the *hph* plasmid described in Experimental Procedures.

with the same construct lacking the *FTF1* fragment responsible for the formation of double-stranded RNAi (dsRNAi) were as virulent as the original wild-type strains. These results demonstrate that dsRNAi transcribed from both promoters in the transforming constructs induces gene silencing in *F. oxysporum*. However, the efficiency of gene silencing is greater in the *phaseoli* strain FOP-SP1 than in the *lycopersici* strain 4287. The comparison of the virulence exhibited by the *FTF2*$^{-}$ mutants and the best *FTF* attenuated mutants indicates a greater contribution to virulence of functional *FTF1* paralogues with respect to *FTF2*.

FTF1 does not enhance virulence in a WV strain

The virulence of transformants SP1*FTF*RNAi.2 and SP1*FTF*RNAi.16, as displayed in common bean inoculation assays, reaches final CIAT scale indices similar to those recorded for WV *F. oxysporum* f. sp. *phaseoli* isolates (Alves-Santos *et al.*, 2002b), although the progression of the disease is different. To test whether *FTF1* alone could be responsible for the virulence differences between HV and WV strains, the WV strain FOP-SP4 was transformed with a functional copy of *FTF1* driven by promoter *Pphasl14257* (Niño-Sánchez *et al.*, 2015). Two transformants were selected for inoculation assays. One showed a single integration event, whereas the other showed three integration events, as demonstrated by PCR and Southern analysis (Fig. S5, see Supporting Information). Therefore, the transformants have genetic backgrounds equivalent to FOP-SP4 plus one or three *FTF1* functional native copies. The latter has a more similar copy number to the HV strain FOP-SP1. The results of inoculation assays of common bean plants showed no significant differences between any of the transformants and the FOP-SP4 wild-type (Fig. S6A, see Supporting Information). Transformants of FOP-SP4 were also obtained with a construct, p*PgpdA::FTF1*, which harboured the same copy of *FTF1* formerly used, driven by the constitutive promoter *PgpdA*. Six transformants were selected and used to inoculate common bean plants, but none showed significant changes in virulence (Fig. S6B).

To assess whether the above results were the consequence of a lack of transcription of the transformed *FTF1* copy in the genetic FOP-SP4 background, semi-quantitative RT-PCR analyses were performed. An *FTF1* cDNA band could be amplified from RNAs obtained from plants inoculated with transformants harbouring the *FTF1* copy driven either by the native or constitutive promoter at 14 days post-infection (Fig. S6C). Therefore, the WV strain is able to produce *FTF1* transcripts, although with no effect on virulence.

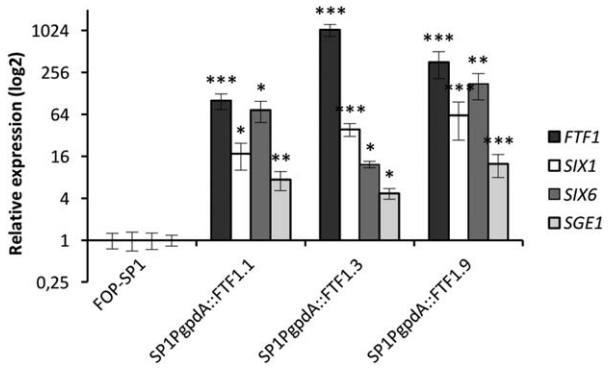

Fig. 6 Gene expression analysis in *Fusarium oxysporum* f. sp. *phaseoli PgpdA::FTF1* transformants. Gene expression of the effector-encoding genes *SIX1* (*Secreted In Xylem 1*) and *SIX6* (*Secreted In Xylem 6*) and the transcription factors *SGE1* (*Six Gene Expression 1*) and *FTF1* (*Fusarium transcription factor 1*) was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in three different *FTF1* constitutive transformants (SP1*PgpdA::FTF1*.1, SP1*PgpdA::FTF1*.3 and SP1*PgpdA::FTF1*.9). The value 1.0 was denoted for the transcript level of the four genes in the wild-type strain FOP-SP1. Error bars indicate the standard deviations in three independent biological experiments. The expression differences between each transformant and the wild-type strain were tested using one-way analysis of variance (ANOVA) followed by Dunnett's test, and are indicated by $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

FTF1 is involved in the regulation of virulence factors

The former results indicate that other virulence factors, apart from *FTF1*, are present in HV and absent in WV strains. It has been shown that the effector genes *SIX1* and *SIX6* share the same expression pattern as *FTF1* during infection of common bean (Niño-Sánchez *et al.*, 2015). Our analyses of the FOP-SP4 genome indicate that this WV strain is devoid of both *SIX1* and *SIX6* genes. Therefore, the virulence of HV strains could be explained if transcription factor FTF1 acts as a positive regulator of effector genes, such as *SIX1* and *SIX6*.

To test this hypothesis, we first analysed the expression of *SIX1* and *SIX6* in *PgpdA::FTF1* transformants of HV strain FOP-SP1, which constitutively express *FTF1*. We included in the analysis the gene coding for the transcripton factor *SGE1* (*Six Gene Expression 1*), as it has been proposed to regulate the expression of some *SIX* genes in *F. oxysporum* f. sp. *lycopersici* (Michielse *et al.*, 2009b). RT-qPCR analyses performed with RNA obtained from three independent transformants grown for 72 h in liquid culture showed a drastic induction of *FTF1*, which correlated with the induction of the three genes analysed (Fig. 6). These results support those previously obtained, which correlated *FTF1*, *SIX1* and *SIX6* expression during host colonization (Niño-Sánchez *et al.*, 2015).

Second, we took advantage of the RNAi transformants obtained from the *phaseoli* and *lycopersici* wild-type strains to compare the expression during host colonization. Figure 7 shows the expression of the three genes analysed in common bean plants

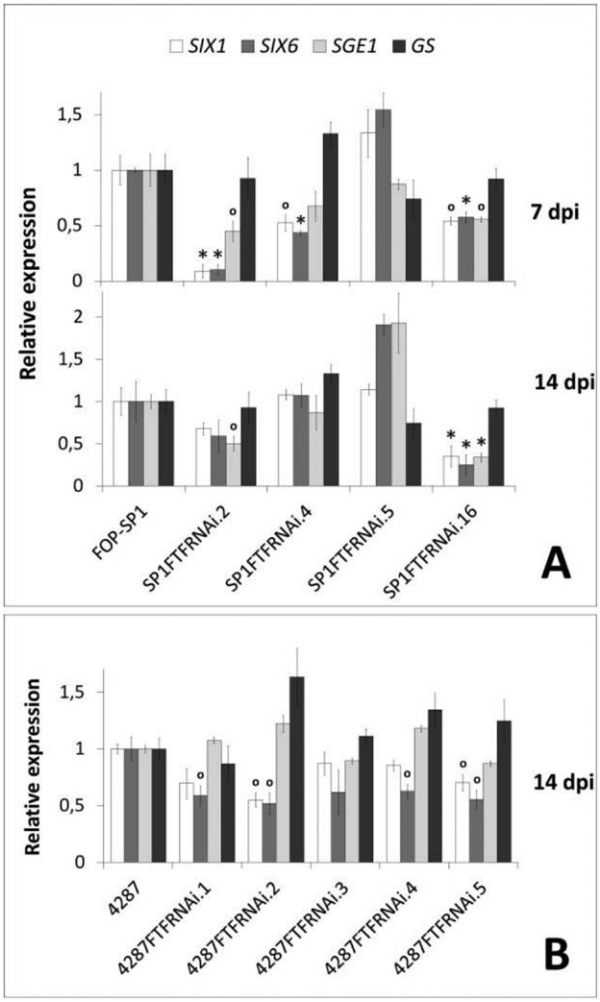

Fig. 7 Gene expression analysis in *Fusarium oxysporum* attenuated transformants. The expression of the effector-encoding genes *SIX1* (*Secreted In Xylem 1*) and *SIX6* (*Secreted In Xylem 6*), the transcription factor *SGE1* (*Six Gene Expression 1*) and the glutamine synthetase gene (*GS*), used as control, was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in inoculated common bean plants at 7 and 14 days post-inoculation (dpi) (A) and inoculated tomato plants at 14 dpi (B). FOP-SP1 and 4287 are *F. oxysporum* f. sp. *phaseoli* and *lycopersici* wild-type strains, respectively; SP1*FTF*RNAi.2, SP1*FTF*RNAi.4, SP1*FTF*RNAi.5 and 4287*FTF*RNAi.1–5 are hygromycin-resistant silenced transformants; SP1*FTF*RNAi.16 is a phleomycin-resistant silenced transformant. The value 1.0 was denoted for the transcript level of all genes in the wild-type strains FOP-SP1 and 4287. Error bars indicate the standard deviations obtained in three independent biological experiments. The differences between each transformant and the wild-type strain were tested using one-way analysis of variance (ANOVA) followed by Dunnett's test, and are indicated by $*P < 0.05$ and $°P < 0.1$.

(Fig. 7A) and tomato plants (Fig. 7B) inoculated with the same transformants, harbouring the silencing construct, as described previously. The greatest reduction in *SIX1* and *SIX6* expression ($P < 0.05$) corresponds to plants inoculated with SP1*FTF*RNAi.2

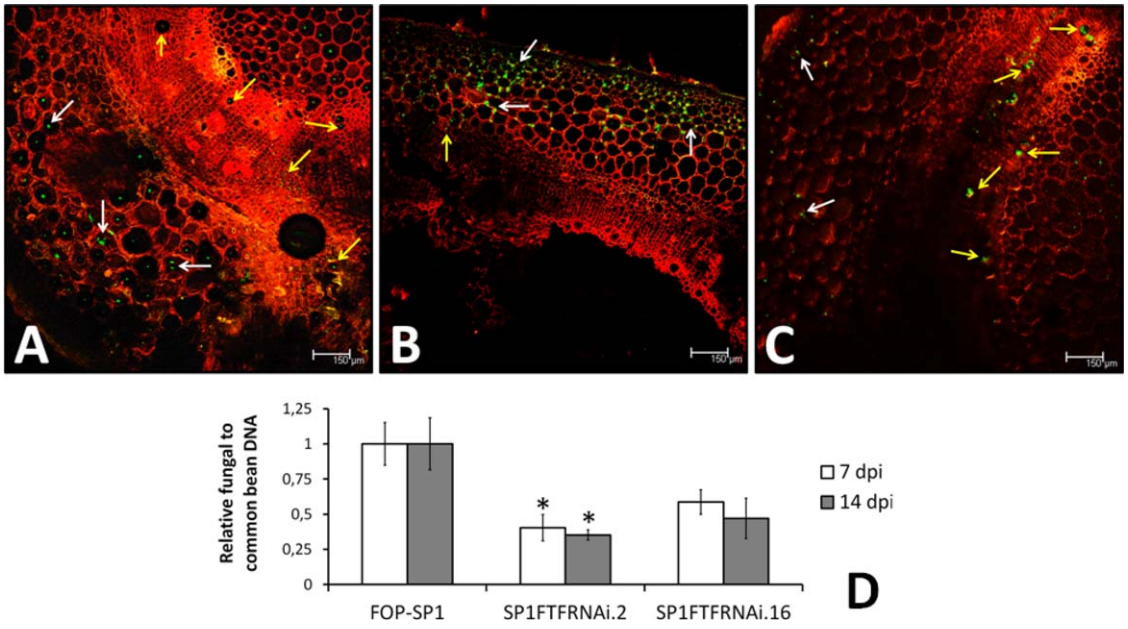

Fig. 8 Plant colonization by green fluorescent protein (GFP)-expressing transformants of *Fusarium oxysporum* f. sp. *phaseoli*. Cross-sections of hypocotyls of common bean plants inoculated with GFP-expressing transformants derived from the SP1FTFRNAI.16 attenuated strain (A), the weakly virulent (WV) strain FOP-SP4 (B) (Niño-Sánchez *et al.*, 2015) and the highly virulent (HV) strain FOP-SP1 (C) were examined by confocal laser microscopy. Hyphae growing in the parenchyma region are indicated by white arrows, whereas hyphae growing inside the xylem vessels are indicated by yellow arrows. Fungal biomass was quantified by quantitative polymerase chain reaction (qPCR) in plants inoculated with FOP-SP1 and two attenuated transformants (SP1*FTF*RNAi.2 and SP1*FTF*RNAi.16). Fungal DNA relative to common bean DNA was measured by assaying the fungal *EF1α* gene and the plant *actin* gene by qPCR using DNA extracted from the root crown at 7 days post-inoculation (dpi) and hypocotyls at 14 dpi. All measurements were referred to the arbitrary value of unity obtained during FOP-SP1 colonization. The differences between each transformant and the wild-type strain were tested using one-way analysis of variance (ANOVA) followed by Dunnett's test, and are indicated by $*P < 0.05$.

(7 dpi) and SP1*FTF*RNAi.16 (14 dpi), the transformants that showed the minimum expression of *FTF1*. SP1*FTF*RNAi.4 also showed a clear reduction in *SIX6* expression at 7 dpi ($P < 0.05$) and intermediate levels of expression of the other genes, in agreement with the intermediate expression shown by the *FTF* genes (see Fig. 4I). Transformant SP1*FTF*RNAi.5 showed no reduction in either *FTF1* or the three genes analysed. Some of the best silenced transformants in *FTF1* derived from the *lycopersici* wild-type strain 4287, 4287*FTF*RNAi.2, 4287*FTF*RNAi.4 and 4287*FTF*RNAi.5, also showed the greatest reduction in the expression of the effector genes. The expression of the gene encoding the transcription factor SGE1 was significantly reduced in the best silenced transformants of the *phaseoli* strain, in a similar manner to the effector genes analysed. This result, together with the induction observed in the transformants constitutively expressing *FTF1*, demonstrates that *SGE1*, *SIX1* and *SIX6* are under positive regulation by FTF1, either directly or indirectly. The results obtained with the *FTF*-silenced *lycopersici* strain indicate that *SIX1* and *SIX6* are under FTF1 positive regulation, but are less conclusive concerning *SGE1*.

Host plant colonization by silenced strains

In a former study, we showed that WV and HV strains of *F. oxysporum* f. sp. *phaseoli* not only differ in the severity of symptoms caused in the host plant (virulence), but also in the colonization pattern: HV strains accumulate more mycelium in infected tissues and are almost exclusively restricted to vascular vessels (Niño-Sánchez *et al.*, 2015).

To analyse the morphological pattern of host colonization by the silenced transformants, one transformant, SP1*FTF*RNAi.16, was transformed with a construct harbouring the *GFP* (*green fluorescent protein*) gene under the control of the constitutive promoter *PgpdA*. After checking for the correct integration and expression of *GFP* (data not shown), one of the SP1*FTF*RNAi.16-*GFP* transformants was selected for inoculation tests. This silenced transformant displayed a colonization pattern resembling that exhibited by the WV strain FOP-SP4, characterized by extensive growth around the parenchymal cells of the cortex and limited growth inside the vascular vessels (Fig. 8A,B), in contrast with the pattern observed in plants inoculated with FOP-SP1 (Fig. 8C). The proportion of colonized vessels measured by image analysis was 31.59% ± 4.89%, a value closer to that obtained for FOP-SP4 (11.39% ± 7.03%) than to that obtained for FOP-SP1 (81.06% ± 8.8%) (Niño-Sánchez *et al.*, 2015). The quantification of fungal biomass accumulation in infected plants, measured by RT-qPCR, showed that silenced transformants accumulate less mycelium than the wild-type HV strain FOP-SP1 (Fig. 8B). The largest difference was observed at 7 dpi, in agreement with the

observations made in the above-mentioned study. Again, the phenotype showing the most significant difference from the wild-type strain was displayed by SP1*FTF*RNAi.2, one of the most attenuated transformants.

Both results indicate that *FTF* knockdown transformants obtained in an HV genetic background show a host colonization pattern similar to that displayed by the WV strain FOP-SP4, which is devoid of *FTF1* copies.

DISCUSSION

The expansion of some gene families encoding transcription factors in *F. oxysporum* is a striking question that addresses the genetic bases of virulence and host specificity in this species complex. In this study, we analysed the *FTF* gene expansion, composed of the core genome *FTF2* gene and several paralogues of the LS *FTF1* gene. *FTF2* is well conserved in all filamentous fungi. A single copy of this gene is present in all genomes available to date in FOSC, and also in sets of isolates experimentally found to be non-pathogenic, WV and HV to common bean plants. The *F. graminearum* mutant seems to be altered only in ascospore production and discharge (Son *et al.*, 2011), but this phenotype would not be relevant in *F. oxysporum* as this fungus does not seem to reproduce sexually. We found that *FTF2* mutants of *F. oxysporum* do not display apparent growth or sporulation abnormalities in solid and liquid culture, respectively, but show a slight reduction in virulence. In contrast, the *FTF1* paralogues are exclusive to *F. oxysporum* and can be found in a variable number of copies, which is related both to the forma specialis analysed and the virulence displayed, as shown in this work and in previous reports (Alves-Santos *et al.*, 2002a; Schmidt *et al.*, 2013; de Vega-Bartol *et al.*, 2011). Additional lines of evidence point to the role of these paralogues in virulence, such as the expression during plant infection (Niño-Sánchez *et al.*, 2015; Ramos *et al.*, 2007) and the correlation between the copy number and virulence displayed (de Vega-Bartol *et al.*, 2011). However, direct confirmation of the role of *FTF1* as a virulence factor is lacking because of the experimental problems involving the construction of deletion mutants in all the paralogues. We have successfully used RNA-mediated gene silencing to attenuate the expression of the *FTF* genes, and found that mutants most effectively silenced (less than 50% of *FTF1* expression) show an important reduction in virulence. Constructs expressing dsRNA complementary to target sequences were obtained by placing a fragment of the *FTF1* gene between two promoters in opposite orientations, following the design successfully deployed in other fungi (Ullán *et al.*, 2008). The sense and antisense strands form a dsRNA that triggers gene silencing (Lee *et al.*, 2002; Wang *et al.*, 2003). The presence of a unique *Nco*I cloning site between the two strong promoters (Ullán *et al.*, 2008) allows for easy insertion of DNA fragments from the genes of interest. This feature implemented in a binary vector harbouring an antibiotic resistance cassette for selection (we have successfully used both the *hph* and *ble* genes) results in a system for easy gene silencing in any strain of *F. oxysporum*. This system would be a useful tool to facilitate the functional analysis of gene expansions, such as that described here, where the deletion of all the copies is not feasible. The correlation between the attenuation of gene expression and reduction in virulence was observed in both the *phaseoli* and *lycopersici* transformants, although, for unknown reasons, gene silencing is more effective in transformants obtained from FOP-SP1 than in those obtained from strain 4287. Silencing efficiency may show variations between strains, as reduced attenuation of gene expression has also been reported in *F. oxysporum* f. sp. *conglutinans* 5176, although, in this case, the silencing method relied on the expression of hairpin RNA transgenes (Schumann *et al.*, 2013). In addition, it is worth noting that the number of copies of *FTF1* is higher in 4287 than in FOP-SP1. The reduction in virulence shown by the attenuated mutants is proportionally greater than that shown by the *FTF2*$^{-}$ mutants, supporting a more important role in virulence for the *FTF1* paralogues than for the *FTF2* core genome gene.

The best-described TF gene expansions involved in the pathogenicity of *F. oxysporum* are *EBR1*, with the four paralogues *EBR2*, *EBR3*, *EBR4* and *EBR5*, and *FOW2* (FOXG_06378), with two paralogues, FOXG_12458 and FOXG_21393. The deletion of *EBR1* causes a minor reduction in virulence towards tomato plants and reduced radial growth in complete medium (Jonkers *et al.*, 2013), which is also observed in the deletion mutants obtained in *F. graminearum* (Zhao *et al.*, 2011). The mutant phenotype observed in *F. oxysporum* f. sp. *lycopersici* when *EBR1* is deleted is substantially restored by a copy of *EBR2* placed under the control of the *EBR1* promoter. The gene predominantly expressed, both during culture and tomato colonization, is *EBR1*. When *EBR1* is deleted, the majority of transcripts detected during growth in complete medium correspond to *EBR3* and, to a lesser extent, to *EBR2*; the opposite occurs during tomato colonization (Jonkers *et al.*, 2013). Taken together, these results indicate that *EBR1* and the paralogues *EBR2* and *EBR3* have redundant roles during both saprophytic and *in planta* growth, whereas *EBR4* and *EBR5* are barely expressed in both conditions. *EBR1* is located in the core genome, whereas the copies of *EBR2*, *EBR3* and *EBR4* are located in LS chromosomes. Similarly, copy FOXG_06378 of *FOW2* is located in the core genome (chromosome 2), whereas the two paralogues are located in LS chromosomes (FOXG_12458 in chromosome 3 and FOXG_21393 in chromosome 6). Deletion of *FOW2* in *F. oxysporum* f. sp. *melonis* causes a complete loss of pathogenicity (Imazaki *et al.*, 2007), whereas *FOW2* insertional mutants in *F. oxysporum* f. sp. *lycopersici* show a decrease of 50% relative to the wild-type level of virulence (Michielse *et al.*, 2009a), which suggests that insertions may have occurred in the paralogues.

None of the above-described gene expansions have been shown to be involved in the main characteristic of *F. oxysporum*, its ability to grow and spread through the vascular system of the plant host. On the contrary, the phenotypic features of transformants attenuated in *FTF* expression strongly suggest a role of the *FTF1* paralogues in vascular colonization. In addition to the reduction in virulence demonstrated in the pathogenicity assays, *FTF*-silenced transformants show: (i) lower accumulation than HV strains of fungal biomass in infected host tissues; (ii) a change in the colonization pattern, from almost exclusively vascular to a mixture of parenchymal and vascular; and (iii) the majority of fungal biomass (68%) accumulates around the parenchymal cells instead of inside the xylem vessels. These three features are also main differences that distinguish WV strains from HV strains of *F. oxysporum* f. sp. *phaseoli*. As shown in previous studies, HV strains are true and almost exclusive vascular colonizers that highly express the *FTF1* transcription factor during plant infection, whereas WV strains are better colonizers of parenchyma and lack the *FTF1* paralogues (Niño-Sánchez *et al.*, 2015; Ramos *et al.*, 2007). Furthermore, the presence of extra *FTF1* paralogues correlates with enhanced virulence when SV strains of *F. oxysporum* f. sp. *phaseoli* infect *P. coccineus* L. (de Vega-Bartol *et al.*, 2011). Therefore, we propose that *FTF1* paralogues encode a transcription factor required for enhanced virulence and heavy colonization of the host vascular system.

The *FTF* genes encode transcription factors of the $Zn(II)_2Cys_6$ binuclear cluster DNA-binding type (Todd and Andrianopoulos, 1997). If the expression of *FTF1* paralogues, or *FTF1* together with *FTF2*, is needed for effective colonization and spread through the xylem vessels, their most attractive targets would be genes whose products are involved in the vascular lifestyle of *F. oxysporum*. The best-known groups of molecules that work as specialized pathogenicity factors in *F. oxysporum* are the small, cysteine-rich proteins encoded by the *SIX* genes (Michielse and Rep, 2009). *SIX* genes play an important role in the determination of host specificity and the establishment of gene-for-gene interactions between *F. oxysporum* f. sp. *lycopersici* and its tomato host (Houterman *et al.*, 2008; Rep *et al.*, 2004). We have found direct evidence that strongly suggests that *SIX1* and *SIX6* genes, both present in the genome of HV *phaseoli* strains, but absent in the genome of WV strains, are activated by the FTF1 transcription factor. First, mutants that constitutively express *FTF1* show enhanced *SIX1* and *SIX6* expression. Second, *FTF*-silenced transformants show reduced expression of both genes. These results support the correlation previously found between *FTF1* up-regulation during host colonization and increased expression of *SIX1* and *SIX6* (Niño-Sánchez *et al.*, 2015). It has been reported that *SIX* expression is dependent on the expression of another transcription factor encoded by the gene *SGE1*, as the expression of several *SIX* genes is lost in a deletion mutant of *SGE1* (Michielse *et al.*, 2009b). Our results show that *SGE1* is also up-regulated in the *phaseoli* strain constitutively expressing *FTF1*, whereas *SGE1* expression is down-regulated in *FTF*-silenced strains of *F. oxysporum* f. sp. *phaseoli*. Therefore, *SGE1* is also likely to be under the control of *FTF1*, at least in *F. oxysporum* f. sp. *phaseoli*, thus making the *FTF1* transcription factor a regulator of effector expression.

The variability shown by *FTF* gene expansion, with up to seven similar genes in strain 4287, is higher than in the case of *EBR* and *FOW* expansions. This range of variability, together with the finding that some paralogues are probably not functional because of structural reasons (this work), suggests that the *FTF* gene family has evolved by gene duplication of an ancestral unique gene, most probably *FTF2*, and the subsequent accumulation of mutations in the duplicated copies. The *FTF1* paralogues are located in LS regions enriched in transposons (Ma *et al.*, 2010; Ramos *et al.*, 2007; Schmidt *et al.*, 2013), whose movement could have originated the duplications. The data presented here demonstrate that some of these mutations have determined a functional specialization of the *FTF1* paralogues, which is closely related to the ability to colonize the vascular system.

EXPERIMENTAL PROCEDURES

Fungal strains and culture conditions

The *F. oxysporum* f. sp. *phaseoli* strains FOP-SP1 (HV) and FOP-SP4 (WV) (Alves-Santos *et al.*, 1999, 2002a; Armstrong and Armstrong, 1975; Booth, 1971), and the *F. oxysporum* f. sp. *lycopersici* race 2 strain 4287 (Di Pietro *et al.*, 1998), were used in this study. All strains were grown as described previously (Alves-Santos *et al.*, 1999; de Vega-Bartol *et al.*, 2011). Fungal cultures were established from frozen mycelia stored on 25% glycerol v/v at −80 °C, and incubated at 25 °C with continuous light for 1 week (solid medium) or 5 days at 120–180 rpm (liquid cultures).

Pathogenicity tests

Inoculation of *P. vulgaris* L. cv. Blanca Riñón with conidia from *F. oxysporum* strains and transformants was carried out as described previously (Alves-Santos *et al.*, 1999). After inoculation, the plants were transferred to pots and further incubated in a glasshouse for regular pathogenicity assays, or to 50-mL Falcon® tubes filled with PGM (Plant Growth Medium) solution, covered with foil and incubated as described previously (Niño-Sánchez *et al.*, 2015). Inoculation of tomato cultivar Money Maker was performed as described previously (Di Pietro *et al.*, 1998). Plant infection tests were repeated three times in a randomized design and a total of 60 plants were analysed. Disease severity was measured by assessment of the disease symptoms recorded according to the CIAT scale (Alves-Santos *et al.*, 1999; Pastor-Corrales and Abawi, 1987), weighing the mass of the aerial part of inoculated plants, quantification of the DPR (García-Sánchez *et al.*, 2010) and evaluation of the affected vascular vessels according to the tomato disease index (Rep *et al.*, 2004).

Isolation of *FTF2*

A genomic library was constructed with DNA from strain FOP-SP4 as described previously (Ramos *et al.*, 2007). To identify the *FTF2* gene, 4000 recombinant phages were screened using the probe B285-A5, which contains part of the promoter and the 5′ coding region of *FTF1*. DNA purified from the positive phage clones was digested with different restriction enzymes and subjected to Southern blot analysis with several probes derived from the coding region of *FTF1*. DNA fragments corresponding to hybridization bands were subcloned into pBluescripts KS+ vector and sequenced using the same primers as designed for sequencing of the *FTF1* gene (Ramos *et al.*, 2007). The nucleotide sequence of gene *FTF2* is available in GenBank under Accession No. JN167165.

Construction of silencing and disruption vectors and generation of mutants

Plasmid p*FTF2*-KO was constructed to inactivate the gene *FTF2* in FOP-SP1. To prevent the partial deletion of the FOXG_09391 ORF, contiguous to the 3′ end of *FTF2* (locus FOXG_09390), primers Inac-FTF2-A3 and Inac-FTF2-A4 were designed to PCR amplify a 776-bp DNA fragment from the 3′ end of the *FTF2* coding region. An 844-bp DNA fragment was amplified from the 5′ flanking region of *FTF2* using primers Inac-FTF2-O1 and Inac-FTF2-O2. Both fragments were PCR amplified using DNA from strain FOP-SP4 as template and cloned into pRF-HU2 digested with *Pac*I and *Nt.Bbv*CI USER^{TM} enzymes (Frandsen *et al.*, 2008).

Plasmids p*FTF*Phleo-RNAi and p*FTF*Hyg-RNAi were designed on the basis of the dsRNA expression cassette from pJL43-RNAi (Ullán *et al.*, 2008) and the binary vector pRF-HU2 (Frandsen *et al.*, 2008). For plasmid p*FTF*Phleo-RNAi construction, first the hygromycin resistance cassette in pRF-HU2 was excised by digestion with restriction enzymes *Pac*I and *Nt.Bbv*CI. Then, a PCR fragment containing the P*pbcC* promoter of the *Penicillium chrysogenum pcbC* gene and the P*gpdA* promoter of the *Aspergillus nidulans* glyceraldehyde 3-phosphate dehydrogenase gene, together with the *ble* gene under the control of the glutamate dehydrogenase promoter (P*gdh*) of *Aspergillus awamori*, was amplified using pJL43-RNAi as template and primers RNAi-U1 and RNAi-U4. This 3406-bp amplicon was cloned into pRF-HU2 digested with *Pac*I and *Nt.Bbv*CI USER^{TM} enzymes (Frandsen *et al.*, 2008), yielding plasmid pPhleo-RNAi. Primers SilFTF2F-NcoI and SilFTF2R-NcoI were used to amplify a 462-bp fragment, containing a conserved *FTF* sequence, using DNA from FOP-SP4 as template. Finally, the 474-bp amplicon and plasmid pPhleo-RNAi were digested with restriction enzyme *Nco*I and ligated using T4 DNA ligase to obtain p*FTF*Phleo-RNAi. To obtain p*FTF*Hyg-RNAi, primers RNAi-User1 and RNAi-User2 were used to PCR amplify a 1.7-kb DNA fragment employing p*FTF*Phleo-RNAi as template. This amplicon contains the double-stranded expression cassette with the *FTF* sequence cloned in the internal *Nco*I site. The 1.7-kb DNA fragment was cloned into pRF-HU digested with *Pac*I and *Nt.Bbv*CI USER^{TM} enzymes.

Complementation of *FTF2*$^{-}$ mutants was performed by transformation with plasmid p*FTF2*, which contains the wild-type allele *FTF2* from strain FOP-SP4 and the *ble* gene for phleomycin resistance. First, plasmid pRF-HU was digested with the enzymes *Apa*I and *Sca*I to excise a fragment containing the *hph* gene. Then, a fragment containing the phleomycin resistance cassette was PCR amplified using primers PhleoF-ApaI and PhleoR-ScaI and DNA from plasmid pJL43-RNAi. This amplicon was ligated to the formerly digested plasmid pRF-HU, thus replacing the *hph* gene by the *ble* gene. Finally, a 4717-bp amplicon containing the wild-type allele *FTF2* was obtained using primers PromFTF2USER-F and TerFTF2USER-R and DNA from strain FOP-SP4 as template, and ligated to the formerly obtained plasmid digested with *Pac*I and *Nt.Bbv*CI USER^{TM} enzymes.

Plasmid p*FTF1* was constructed to express the gene *FTF1* in the strain FOP-SP4 under the control of its native promoter. A DNA fragment was amplified using primers B310User and M40Auser, and DNA from FOP-SP1 as template. The 4890-bp amplicon contains the *FTF1* paralogue homologous to FOXG_14257 in *F. oxysporum* f. sp. *lycopersici*, and contains the ORF together with 670 bp of the promoter region and 1 kb of the 3′ nontranslated region. The amplicon was ligated to pRF-HU digested with *Pac*I and *Nt.Bbv*CI USER^{TM} enzymes. In a similar way, plasmid p*PgpdA::FTF1* was constructed to express the gene *FTF1* under the constitutive control of the *PgpdA* promoter and the *TrpC* terminator harboured by pRF-HUE. Primers FTF1UserF and FTF1UserR were used in PCRs with DNA from FOP-SP1 as template. The amplicon contains the complete ORF of the *phaseoli* paralogue homologous to FOXG_14257 in *F. oxysporum* f. sp. *lycopersici*, and was ligated to pRF-HUE digested with *Pac*I and *Nt.Bbv*CI USER^{TM} enzymes (Frandsen *et al.*, 2008).

All the constructs were used to genetically transform *F. oxysporum* employing the *Agrobacterium tumefaciens*-mediated transformation procedure as described previously (Mullins *et al.*, 2001; Ramos *et al.*, 2007).

Construction of GFP-expressing strains

Transformants of *F. oxysporum* f. sp. *phaseoli* attenuated in the expression of *FTF1* and *FTF2* were genetically transformed to express GFP and verified for normal pathogenicity, as described previously (Niño-Sánchez *et al.*, 2015).

Confocal laser microscopy

The plants inoculated with FOP-SP1, FOP-SP4 and the attenuated transformants expressing GFP were maintained in hydroponic cultures and examined each day for a period of 21 days after infection, as described previously (Niño-Sánchez *et al.*, 2015). The quantification of xylem vessel colonization was performed with the help of ImageJ and carried out as described previously (Niño-Sánchez *et al.*, 2015).

Nucleic acid manipulations

Genomic DNA was extracted from *F. oxysporum* mycelium according to the procedures described previously (Alves-Santos *et al.*, 1999, 2002b; Ramos *et al.*, 2007). RNA was extracted from common bean and tomato plants at different times after inoculation with *F. oxysporum* strains and transformants. Roots and hypocotyls were cut and immediately frozen at 80 °C and ground in a pestle and mortar under liquid nitrogen. Southern blots were carried out as described previously (Ramos *et al.*, 2007). DNA probes were labelled with digoxigenin-dUTP (Roche Diagnostics, Basel, Switzerland) by the PCR method using Biotools DNA Polymerase (Biotools SA, Madrid, Spain). Prehybridization, hybridization, washing and detection were performed using a chemiluminescent detection procedure employing CDP-Star (Roche Diagnostics) according to the manufacturer's recommendations.

Real-time quantitative analysis of gene expression and fungal biomass quantification

Samples of plants inoculated with *F. oxysporum* were collected and frozen at −80 °C. Total RNA was extracted and DNAase was treated using the SV Total RNA Isolation System Z3105 (Promega, Madison, WI, USA) according to the manufacturer's recommendations. RNA quantification and cDNA synthesis were performed as described previously (Niño-Sánchez *et al.*, 2015). The efficiency of each pair of primers was verified as described previously (Niño-Sánchez *et al.*, 2015). Amplifications were performed in a StepOnePlus™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. The *F. oxysporum EF1α* gene and the common bean actin gene were used as endogenous reference genes. The relative expression levels of each gene were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Three sets of samples from different infection assays were used (biological replicates), two independent cDNA preparations per biological replicate were obtained and three replicates of each cDNA were analysed to calculate the mean and standard deviation. All the primers used in RT-qPCR experiments and in the construction of vectors are listed in Table S2 (see Supporting Information).

Fungal biomass in inoculated plant samples was estimated as described previously (Niño-Sánchez *et al.*, 2015).

Similarity analysis

Similarity searches were performed with the BLAST program against public DNA/protein databases [National Center for Biotechnology Information (NCBI) and Broad Institute]. The alignment of *FTF2* homologous sequences and the comparison of *FTF1* and *FTF2* sequences were made with the help of the Geneious program (Biomatters, Auckland, New Zealand) and its default parameters (IUB cost matrix, neighbour joining, Tamura–Nei distance model) for MUSCLE/CLUSTALW and GBLOCKS. Phylogenetic reconstructions were performed using PhyML and evaluated using a bootstrap test with 100 replications. The motifs, regions and domains of *FTF2* were characterized at the Broad website (www.broadinstitute.org) and annotated with the Geneious program.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website.

Fig. S1 Nucleotide sequence homology between all members of the FTF (*Fusarium transcription factor*) gene family. (A) Percentages of homology between the open reading frames (ORFs). (B) Percentages of homology between the 462-bp fragment used for silencing. The letters in parentheses indicate the type of protein deduced from the ORF of each locus.

Fig. S2 Gene expression analysis of FTF2 (*Fusarium transcription factor 2*) during growth in culture. The expression of the gene FTF2 was measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in mycelia grown in liquid culture (potato dextrose broth, PDB). FOP-SP1 and FOP-SP4 are highly virulent (HV) and weakly virulent (WV) Fusarium oxysporum f. sp. phaseoli wild-types, respectively. The expression measurements for each strain were referred to the arbitrary value of unity obtained after 6 h of growth. Error bars indicate the standard deviations obtained in three independent biological experiments.

Fig. S3 Southern hybridization of *Hind*III-digested DNA from knockout and silenced transformants of *Fusarium oxysporum*. Digested DNA from knockout mutants obtained from strain FOP-SP1 and the mutant complemented with the *FTF2* (*Fusarium transcription factor 2*) wild-type allele, hybridized with probe M18B-M19A (a fragment of the *FTF1* gene) (A, B). Digested DNA from attenuated transformants obtained from strain FOP-SP1 and hybridized with probe SilFTF2F.NcoI-SilFTF2R.NcoI which includes the silencing-inducer *FTF1* fragment (C), probe HphF-HphR which includes the hyg resistance gene (D), and probes HphF-HphR and PhleoF-PhleoR, the latter including the phleo resistance gene (E). Digested DNA from attenuated transformants obtained from strain 4287 and hybridized with probe SilFTF2F.NcoI-SilFTF2R.NcoI (F) and probe HphF-HphR (G). SP1Δ*FTF2*.1–6 are knockout mutants obtained by transformation of strain FOP-SP1; SP1Δ*FTF2-FTF2* is mutant SP1Δ*FTF2*.1 complemented with a wild-type *FTF2* allele; FOP-SP1 and FOP-SP4 are highly virulent (HV) and weakly virulent (WV) strains of *F. oxysporum* f. sp. *phaseoli*, respectively; AB82 is a non-pathogenic *F. oxysporum* strain; SP1*FTF*RNAi.2, SP1*FTF*RNAi.4 and SP1*FTF*RNAi.5 are attenuated mutants obtained by transformation of strain FOP-SP1 with the silencing construct containing the *hph* gene; SP1*FTF*RNAi.16 is an attenuated mutant obtained by transformation of strain FOP-SP1 with the silencing construct containing the *phleo* gene; SP1*FTF*RNAi was obtained by transformation of FOP-SP1 with the backbone of the silencing construct lacking the silencing-inducer fragment; 4287*FTF*RNAi1–5 are attenuated mutants obtained by transformation of strain 4287 with the silencing construct containing the *hph* gene; 4287*FTF*RNAi was obtained by transformation of 4287 with the backbone of the silencing construct lacking the silencing-inducer fragment; 4287 is an *F. oxysporum* f. sp. *lycopersici* race 2 strain.

Fig. S4 Growth in solid culture (potato dextrose agar, PDA) and sporulation in liquid culture (potato dextrose broth, PDB) of *FTF2* (*Fusarium transcription factor 2*) mutants (SP1Δ*FTF2*.1 and SP1Δ*FTF2*.2), an *FTF2* mutant complemented with the *FTF2* wild-type allele (SP1Δ*FTF2-FTF2*), *FTF* attenuated mutants obtained by transformation of strain FOP-SP1 (SP1*FTF*RNAi.2, SP1*FTF*RNAi.4, SP1*FTF*RNAi.5, SP1*FTF*RNAi.16 and SP1*FTF*RNAi) or strain 4287 (4287*FTF*RNAi1-5 and 4287*FTF*RNAi), and the wild-type strains FOP-SP1 and 4287. Measurements were performed after 7 days of growth.

Fig. S5 Southern hybridization of *Hind*III-digested DNA from transformants of *Fusarium oxysporum*. The membrane was hybridized with probe M18B-M19A which includes the *FTF1* (*Fusarium transcription factor 1*) gene (A) and probe HphF-HphR which includes the *hph* gene (B). FOP-SP4 is a weakly virulent (WV) wild-type strain; SP4*FTF1*.1 and SP4*FTF1*.2 are

strains derived from FOP-SP4 by transformation with a plasmid containing the native *FTF1* gene, and contain one copy and three copies, respectively, of *FTF1*. Size markers (kb) are indicated on the left.

Fig. S6 Fusarium wilt induced in common bean plants by *Fusarium oxysporum* f. sp. *phaseoli* strains expressing the *FTF1* (*Fusarium transcription factor 1*) gene. Disease symptoms were measured according to the Center for Tropical Agriculture (CIAT) scale in plants inoculated with transformant strains (SP4*FTF1*.1 and SP4*FTF1*.2) harbouring copies of the native *FTF1* gene (A) or with transformants harbouring the *FTF1* open reading frame (ORF) constitutively expressed by the *gpdA* promoter (SP4*PgpdA::FTF1*.1–6) (B). All the transformants were derived from the weakly virulent (WV) FOP-SP4 strain. (C) Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out using RNA extracted from plant hypocotyls at 14 days post-inoculation (dpi). FOP-SP1 is a highly virulent (HV) strain.

Table S1 Growth and sporulation of wild-types, knockout and attenuated transformants of *Fusarium oxysporum*.

Table S2 Oligonucleotides used in this study.