New Virulence Groups in *Fusarium oxysporum* f. sp. *phaseoli*: The Expression of the Gene Coding for the Transcription Factor *ftf1* Correlates with Virulence

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ABSTRACT

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Fusarium oxysporum f. sp. *phaseoli* strains isolated from runner bean plants showing Fusarium wilt symptoms were characterized. The analysis of the genetic diversity of these strains and the comparison with strains formerly isolated from diseased common bean plants grown in the same region of Spain indicated a close genetic similarity among them. Pathogenicity assays carried out on runner bean plants showed virulence differences that allowed the classification of these strains into three groups: super virulent, highly virulent, and weakly virulent. However, all

the analyzed strains behaved as highly virulent when inoculated on common bean plants, indicating that virulence is specific of the host-pathogen interaction. We also analyzed the number of copies and expression of the gene encoding the transcription factor *ftf1*, which has been shown to be specific of virulent *F. oxysporum* strains and highly up-regulated during plant infection. In planta real-time quantitative polymerase chain reaction expression analysis showed that expression of *ftf1* was correlated with the degree of virulence. The comparative analysis of the polymorphic copies of *ftf1* detected in the strains here characterized and those detected in the genome sequence of *F. oxysporum* f. sp. *lycopersici* strain 4287 indicates that some of the copies are likely nonfunctional.

Additional keywords: pathogenicity, *Phaseolus coccineus*.

Fusarium oxysporum Schlechtend.:Fr. is an anamorphic species complex with considerable morphological and physiological variation (39). This fungus is ubiquitous in soils worldwide and some strains are pathogenic, producing diseases of economically important plant hosts. Symptoms are characterized by chlorosis of leaves, necrosis of the vascular system, and general wilt and death of the colonized plant. Strains may be classified into *formae speciales* based on host specificity (9,23,24), making the *F. oxysporum* species complex a very attractive system for the study of the relationships between pathogenicity, virulence, and host specificity. While pathogenicity and host specificity in *F. oxysporum* have been the subject of numerous studies and their molecular determinants are beginning to be known (19,25,36), the molecular basis of virulence and the relationship between virulence and host specificity remains largely unknown (48).

Fusarium wilt caused by *F. oxysporum* f. sp. *phaseoli* is the most important disease that affects the common bean cultivars grown in El Barco de Avila (Castilla y León, west-central Spain) (15,50), which have achieved an excellent reputation due to their gastronomic characteristics. In this area, runner bean (*Phaseolus*

coccineus L.) is also grown although to a lesser extent than common bean (*P. vulgaris* L.). Given that isolates of *F. oxysporum* f. sp. *phaseoli* are able to colonize several species of the genus *Phaseolus*, the analysis and comparison of *F. oxysporum* f. sp. *phaseoli* isolates collected from both hosts in a limited geographic area, offers a good opportunity to study the molecular determinants of virulence and their expression in different but related hosts. In previous studies, we analyzed the genetic diversity, pathogenicity, and race structure of a collection of *F. oxysporum* f. sp. *phaseoli* strains obtained from diseased common bean plants (2–4). We showed that pathogens from the studied area correspond to a new race and are clearly different from pathogenic strains from other geographic areas, thus suggesting a polyphyletic origin for *F. oxysporum* f. sp. *phaseoli*. The genetic similarities of these collected pathogenic strains, as shown by the analysis of intergenic spacer region polymorphism of ribosomal DNA (IGS haplotypes), electrophoretic karyotypes (EKs) patterns (2), and random amplifications of polymorphic DNA (RAPDs) (4), suggests a common origin for most or all of them. We also found that these strains could be separated into two virulence groups (3), which poses an intriguing question concerning the acquisition and evolution of virulence factors: do isolates of a *forma specialis*, likely sharing a common ancestor, use the same virulence factors to attack their hosts? The simplest answer to this question would be that different virulence groups would use different virulence factors. This conclusion was supported by the discovery that weakly virulent strains lack a transcription factor, named *ftf1* (*Fusarium transcription factor 1*), which is present as a multiple copy gene in highly virulent strains and is dramatically

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up-regulated during the first stages of common bean colonization (42). This gene was identified in the course of a RAPD analysis carried out to determine the genetic diversity of pathogenic and nonpathogenic populations of *F. oxysporum* from common bean fields in Spain (4). *ffl1* encodes a transcription factor containing a Zn(II)-Cys6 binuclear cluster DNA-binding motif, and experimental evidence suggests it is a virulence factor characteristic of *F. oxysporum* virulent strains (42, de Vega-Bartol, *unpublished data*). The presence of multiple copies of *ffl1* offers another solution to the problem of the relationship between virulence groups and virulence factors. A combination of gene expression regulation and copy number regulation of a virulence gene would result in a fine tuning of the degree of virulence and the damage caused to the host, thus allowing for a better correlation between parasite fitness and virulence and more plasticity in host–parasite coevolution. An example of host–pathogen interaction that seems to follow this model is the foliar pathogen of wheat (*Pyrenophora tritici-repentis*), which produces toxin *ToxB* that is encoded by a multiple-copy gene (34,35). It has been shown that *ToxB* transcript is more abundant in virulent race 5 isolates than in weakly virulent race 5 isolates (5).

To test the above mentioned hypothesis we decided to analyze the virulence characteristics and genetic structure of a collection of *F. oxysporum* f. sp. *phaseoli* isolates obtained from runner bean plants, another host for this *forma specialis*, and test whether a correlation could be established between virulence, the copy number of *ffl1*, and the expression of this gene.

MATERIALS AND METHODS

***F. oxysporum* strains.** Isolates were recovered from stems of *P. coccineus* (runner bean) plants that showed typical symptoms of Fusarium wilt. Diseased plants were sampled during two growing seasons (May to October) from several plots around the village of Villafranca de la Sierra in the area of El Barco de Ávila (Ávila, Spain) where local cultivars have been grown for decades. Mycelia suspected to be *F. oxysporum* were identified by microscopic examination of spores and conidiophores. Single conidial isolates were obtained from each isolate and only one isolate obtained from each plant was further analyzed.

F. oxysporum f. sp. *phaseoli* strains FOP-SP01, FOP-SP04, and AB7 isolated from common bean plants have been previously characterized (2,3). Also, *nitM* mutants from a strain of each of the vegetative compatibility groups (VCGs) previously described in *F. oxysporum* f. sp. *phaseoli* were selected for VCG determination. All the isolates used in the present study are listed in Table 1.

Culture media and conidia isolation. Fungal cultures were established from colonies resulting from the subculture of a single conidium stored as previously described (2) on 25% glycerol, vol/vol, at –80°C for long-term conservation. All strains were grown in potato dextrose broth (PDB; Difco Laboratories, Detroit) or potato dextrose agar (PDA; Difco Laboratories) as previously described (2). Cultures were incubated at 25°C with continuous light for 5 days (liquid cultures) or 7 days (solid

TABLE 1. *Fusarium oxysporum* f. sp. *phaseoli* strains used in this work and characterized by vegetative compatibility group (VCG), intergenic spacer (IGS) haplotypes, mating-type (MAT) idiomorphs, and group of virulence (virulence) in *Phaseolus coccineus* (runner bean) and *P. vulgaris* (common bean)^a

Strain	VCG	IGS	MAT	Virulence	Pc-DPR	Pv-DPR	Geographical origin
ATCC18131	0161 ^b	B5 ^b		Highly virulent ^c			South Carolina, USA ^d
Fo 5 BRA (BR5)	0162 ^b	A ^b		Pathogenic			Pernambuco, Brazil ^e
Fo 28 COL (CL28)	0163 ^b			Highly virulent ^c			Colombia ^c
Fo 25 COL (CL25)	0164 ^b	B4 ^b		Highly virulent ^c			Colombia ^d
Fo 10 Ph (GR10)	0165 ^b			Highly virulent ^c			Kastoria, Greece ^f
FOP-SP04	0166 ^b	A ^b		Weakly virulent ^c			Ávila, Spain ^g
FOP-SP01	0167 ^b	A ^b		Highly virulent ^c			Ávila, Spain ^g
FOP-SP03	0168 ^b			Highly virulent ^c			Ávila, Spain ^g
FOP-SP13	0169	A	MAT1-2	Super virulent	9,0 (0,0)	8,4 (0,1)	Ávila, Spain
FOP-SP14	0169	A	MAT1-2	Super virulent	9,0 (0,0)	–	Ávila, Spain
FOP-SP15	0169	A	MAT1-2	Super virulent	9,0 (0,0)	–	Ávila, Spain
FOP-SP16	0169	A	MAT1-2	Super virulent	8,3 (0,7)	–	Ávila, Spain
FOP-SP17	0169	A	MAT1-2	Super virulent	8,5 (0,6)	–	Ávila, Spain
FOP-SP18	0169	A	MAT1-2	Super virulent	9,0 (0,0)	–	Ávila, Spain
FOP-SP19	0169	A	MAT1-2	Super virulent	9,0 (0,0)	–	Ávila, Spain
FOP-SP20	0169	A	MAT1-2	Super virulent	8,9 (0,1)	–	Ávila, Spain
FOP-SP21	0169	A	MAT1-2	Super virulent	9,0 (0,0)	–	Ávila, Spain
FOP-SP22	0169	A	MAT1-2	Highly virulent	7,3 (0,6)	–	Ávila, Spain
FOP-SP23	0169	A	MAT1-2	Highly virulent	6,0 (1,1)	9,0 (0,0)	Ávila, Spain
FOP-SP24	0169	A	MAT1-2	Highly virulent	6,6 (1,6)	–	Ávila, Spain
FOP-SP25	0169	A	MAT1-2	Highly virulent	7,5 (0,9)	8,0 (0,6)	Ávila, Spain
FOP-SP26	0169	A	MAT1-2	Highly virulent	7,8 (0,6)	–	Ávila, Spain
FOP-SP27	0169	A	MAT1-2	Highly virulent	7,9 (0,5)	–	Ávila, Spain
FOP-SP28	0169	A	MAT1-2	Highly virulent	7,4 (0,5)	–	Ávila, Spain
FOP-SP29	0169	A	MAT1-2	Highly virulent	7,0 (0,2)	–	Ávila, Spain
FOP-SP30	0169	A	MAT1-2	Weakly virulent	6,0 (0,7)	–	Ávila, Spain
FOP-SP31	0169	A	MAT1-2	Weakly virulent	4,3 (0,3)	6,8 (1,6)	Ávila, Spain
FOP-SP32	0169	A	MAT1-2	Weakly virulent	5,1 (0,5)	8,9 (0,2)	Ávila, Spain
FOP-SP33	0169	B2	MAT1-2	Highly virulent	6,4 (1,0)	7,9 (1,0)	Ávila, Spain
FOP-SP34	01610	A	MAT1-2	Weakly virulent	3,5 (1,3)	9,0 (0,0)	Ávila, Spain
FOP-SP35	01610	B2	MAT1-2	Weakly virulent	5,1 (1,0)	9,0 (0,0)	Ávila, Spain
FOP-SP36	01611	B2	MAT1-2	Weakly virulent	5,2 (0,5)	8,5 (0,5)	Ávila, Spain
FOP-SP37	01612	B2	MAT1-2	Weakly virulent	3,1 (1,2)	7,8 (0,5)	Ávila, Spain

^a Pc-DPR, disease progression rate in *P. coccineus* (runner bean); or Pv-DPR, disease progression rate in *P. vulgaris* (common bean) as host.

^b Alves-Santos et al., 1999 (2).

^c Alves-Santos et al., 2002 (3). Isolate source.

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media). Mycelia and conidia were separated by filtration through two layers of sterile cheesecloth.

Nitrate medium (MM), nitrite medium (MMN), hypoxanthine medium (MMH), and chlorate medium (MMC) were used to test nit mutant phenotypes (14).

VCG determination. Isolation and characterization of *nit* mutants were performed as previously described (2). Pairings were carried out between *nit1* and *nitM* mutants in MM plates that were incubated at 22°C and scored after 2 weeks. Complementation was indicated by the development of dense aerial growth where the mycelia of the mutants anastomosed to form a heterokaryon. A *nit1* mutant obtained from each analyzed isolate was paired with a *nitM* mutant obtained from an isolate representative of each new VCG, and all pairings were repeated at least once. Finally, *nit1* mutants representative of each new found VCG were paired with *nitM* mutants representative of all formerly described *F. oxysporum* f. sp. *phaseoli* VCGs.

Pathogenicity tests. Inoculations of *P. vulgaris* 'Blanca Riñón' and *P. coccineus* 'Judión de El Barco' with conidia from different strains of *F. oxysporum* f. sp. *phaseoli* were made as previously described (3). All the strains were simultaneously tested in each inoculation experiment to reduce the variability in the cultivar response due to environmental conditions. Plants were grown in an environment-controlled chamber at 23 to 25°C with a 12 h photoperiod of fluorescent light and relative humidity of 60 to 80%. Between 6 and 12 seedlings were inoculated with each of the strains of *F. oxysporum*, and each inoculation was repeated three times. Disease rates were quantified at 1-week intervals using the CIAT scale (40). The disease ratings of all the plants inoculated with the same isolate evaluated in the three experiments were used to calculate the mean disease rating and the standard deviation for each time-point. The disease progression rate (DPR) was measured as previously described (17). Plants inoculated for real-time polymerase chain reaction (PCR) analysis of expression were infected as described, but after infection were transplanted into pots containing a mix of vermiculite and soil (1:3) and grown in a greenhouse.

Nucleic acid manipulations. Genomic DNA was extracted from *F. oxysporum* mycelium after 7 days according to the procedures previously described (2,3,17,42). RNA was extracted

from *P. vulgaris* or *P. coccineus* plants at different times after inoculation with *F. oxysporum* strains. Hypocotyls of at least two plants that were inoculated at the same time were cut and immediately frozen and grinded in a mortar with pestle under liquid nitrogen. RNA was extracted using the QIAGEN RNeasy kit (Qiagen, CA) as recommended by manufacturer, except that initially approximately 100 mg of frozen tissue was recovered in a 1.5-ml tube, mixed with the supplied RTL buffer, and centrifuged for 1 min. Contaminating DNA was removed with Turbo RNA-free Dnase (Ambion Inc., Austin, TX). Southern blots were carried out as described previously (42). DNA probes were labeled with digoxigenin-dUTP by the PCR method using the Biotools DNA polymerase (Biotools SA, Spain) and genomic DNA or cDNA from strain FOP-SP01 as template. Prehybridization, hybridization, washings, and detection were performed using a chemiluminescent detection procedure using CDP-Star (Roche Diagnostics) according to the manufacturer's recommendations. Primer sequences used to obtain the PCR amplicons employed as probes are listed in Table 2.

IGS RFLP analysis. The IGS region of the ribosomal DNA was amplified using primers CNL12 and CNS1 (Table 2) (6) essentially as previously described (2). The amplification conditions included an initial denaturation step (10 min at 95°C), followed by 35 cycles consisting of a denaturation step at 95°C for 30 s, annealing for 1 min at 56°C, and extension for 3 min at 72°C. A final extension step was performed for 7 min at 72°C. A sample (10 µl) of each PCR product was digested with *Clal*, *XhoI*, *AvaI*, or *AccI* restriction enzymes (Roche Diagnostics, Germany) at 37°C during 1 h. Digested DNA was run on 1.2 or 2% (wt/vol) agarose gels, depending on the expected size of the fragments analyzed. The gels were stained in an ethidium bromide solution and restrictions fragments were visualized under UV light.

Mating-type analysis. Mating-type (MAT) was determined by PCR with three sets of primers (Table 2) previously shown to amplify MAT idiomorphs in *GibberellaFusarium* species (52). Amplifications of a 593-bp fragment and a 229-bp fragment from MAT1-2 isolates were performed using oligonucleotide pairs FMATp1/FoMAT2p2 and FoMAT2p10/FMATp2 as primers, respectively; while a 1,475-bp fragment was amplified from MAT1-1 isolates using the oligonucleotides pair FoMAT1-2p1/FoMAT1-2p2 as primers. PCR conditions were 94°C for 10 min, 30 cycles of 94°C for 1 min, and 55.5°C (primers FMATp1/FoMAT2p2), or 60°C (primers FoMAT2p10/FMATp2), or 64°C (primers FoMAT1-2p1/FoMAT1-2p2) for 1 min, and 72°C for 1 min, and a final elongation step of 72°C for 10 min. PCR mixtures (20 µl) contained 1× PCR buffer (Applied Biosystems), 10 ng of fungal DNA, 2.5 mM MgCl₂, 0.25 mM deoxynucleoside triphosphate mix (Applied Biosystems), 5 µM of each primer, and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR amplification products were separated by electrophoresis in 1.2% (wt/vol) agarose gels were stained with ethidium bromide and visualized under UV light.

Pulsed-field gel electrophoresis. Intact chromosomal DNA was prepared from protoplasts as previously described (2). Chromosomal DNA bands were separated in 0.7% (wt/vol) agarose (Seakem Gold, Cambrex Bio Science Rockland) by electrophoresis for 96 h with a contour-clamped homogeneous electric field system (CHEF Mapper, Bio-Rad) at 10°C with continuous circulation of TBE buffer (45 mM Tris-borate and 1 mM EDTA). Buffer was replaced every 48 h. Electrophoretic conditions were 2 V/cm, with pulses ramped from 10 to 30 min and an angle of 106°. Gels were stained in ethidium bromide and visualized under UV illumination. Chromosomal preparations from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Bio-Rad) were used as standards.

Real-time quantitative PCR. Gene expression analyses by means of quantitative PCR (qPCR) were performed using 1 to 2 µg of RNA with POWER SYBR Green RNA-to-Ct 1-Step Kit

TABLE 2. Polymerase chain reaction primers used in this study

Name	Sequence
CNL12 ^a	CTGAACGCCTCTAAGTCAG
CNS1 ^a	GAGACAAGCATATGACTACTG
FoMAT2p10 ^b	GAATAGGGGAAATGCAAAAATCTGA
FMATp2 ^b	AACCTGTGTCTTTAACCTGAGCAT
FMATp1 ^b	GACCTAGTGCAACAAGAAACAAAAGCGAGTG
FoMAT2p2 ^b	GTCACCAAGTCGATCAAGGCTCAAC
FoMAT1-2p1 ^b	GTTTGGCCGATGTGATTGGACTACC
FoMAT1-2p2 ^b	CTACACAACACCCACACTTCTCATCAT
B310 ^c	CAGCATTCATGGATGACATAACGAATTC
A280 ^c	TATACCGGACGGGCGTAGTGACGATG
B5	CGTTCCCTACTAGATCACACCCA
M17A	GTTTGACCACGGGGCGAGTTAC
M18B ^c	CGCAAAAACGCAAGCTCAACTTC
M19A ^c	GCGAGGGGTGCCGAAACAAAGC
QFo-homologia-fwd	GGACATTCATCGCTGCGAA
QFo-homologia-rev	AGGAAAAGGCGCAGTATCCAG
Btub-Fop-fwd	CGACATGAGTGTCTACTTC
Btub-Fop-rev	TGGTACCAGGCTCAAGATCGA
EF1alfa-Q2-Fc ^d	CATCGGCCACGTCGACTCT
EF1alfa-Q2-Rc ^d	AGAACCCAGGCGTACTTGAA
BMd53R ^e	TGCTGACCAAGGAAATTCAG
BMd53R ^e	GGAGGAGGCTTAAGCACAAA

^a Appel and Gordon, 1996 (7).

^b Arie et al., 2000 (8).

^c Ramos et al., 2007 (42).

^d van der Does and Rep, 2008 (48).

^e Blair et al., 2006 (12).

(Applied Biosystems, Foster City, CA) using an ABI Prism 7000 SDS system (Applied Biosystems). DNA contamination of the samples was ruled out by checking the absence of amplicons after qPCR without reverse-transcription enzyme mix (supplied in the kit). A 102-bp fragment of the target gene *ftfl* was amplified using 300 nM of the primers QFo-homologia-fwd and QFo-homologia-rev, and a 134-bp fragment of the endogenous β -*tubulin* gene, used as reference gene, was amplified using 300 nM of the primers Btub-Fop-fwd and Btub-Fop-rev (Table 2). The thermal profile recommended by Applied Biosystems was followed for the amplification procedure (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min), except that the anneal/extend step was performed at 64°C. The presence of only one specific peak was checked in the melting curve (dissociation curve), which was run at 0.5°C increases every 5 s within a range of 55 to 95°C. The qPCR product size was checked by electrophoresis in 2.5% (wt/vol) agarose gels that were stained with ethidium bromide and visualized under UV light. Two sets of samples from different infection assays were used. Three replicas of each sample were analyzed per qPCR run or plate, and each run was repeated two times to verify accuracy. The amplification efficiency of each set of primers was obtained using program LinReg 11.3 (46). Average Ct and standard deviation of the replicas of each strain were calculated for the data analysis. The relative expressions were calculated as described in the comparative Ct or $\Delta\Delta$ Ct method (31,41) as the ratio and deviation between the Ct of the target gene and the endogenous reference in a target sample against a control sample (Supplementary Table 1). The quantification of *F. oxysporum* in inoculated *P. coccineus* plants by real-time PCR was performed as previously described (29). The absolute DNA quantity that corresponds to a given Ct value was interpolated from standard curves, which were constructed using serial dilutions of DNA (from 600 to 0.6 ng/μl) extracted from noninoculated plants or serial dilutions of DNA (from 0.6 to 0.0006 ng/μl) extracted from mycelium grown in PDB medium for 4 days and the resulting Ct values. For the amplification reaction, 1 μl of DNA from each dilution was added to a standard master mix of the kit KAPA SYBR FAST qPCR, as recommended by the supplier (Kapa Biosystems). Amplification of the gene coding for the *F. oxysporum* EF1 translation elongation factor and the runner bean microsatellite BMd53 was performed using primers EF1alfa-Q2-Fc and EF1alfa-Q2-Rc, and BMd53F and BMd53R, respectively (Table 2). These primers were designed to match the standard cycling conditions described above. To compare DNA amounts of strains of the different virulence groups, the average Ct values of each group, representing relative target DNA, were used.

The correlation between the expression of gene *ftfl* and the DPR index for each *F. oxysporum* strain was verified using the Pearson correlation coefficient.

RESULTS

Pathogenicity and virulence. The isolates analyzed were collected during two growing seasons from several plots in the area of El Barco de Avila (Avila, Spain). The 25 strains analyzed were tested for pathogenicity both in runner bean (*P. coccineus*) and common bean (*P. vulgaris*) by means of inoculation assays. All the *F. oxysporum* strains induced the characteristic yellowing and wilting symptoms of Fusarium wilt observed in the field. However, the disease ratings obtained in inoculated runner bean plants indicated that they could be separated into three virulence groups (Fig. 1 and Table 1). Strains FOP-SP13 to FOP-SP21 showed disease ratings close to 9 at 2 weeks after plant infection (most of the plants were dying by 2 weeks) (Fig. 1A). Strains FOP-SP22 to FOP-SP29 and FOP-SP33 showed disease ratings above 7 in the third or fourth week after inoculation (Fig. 1B). Finally, a third group of strains, FOP-SP30 to FOP-SP32 and FOP-SP34 to FOP-

SP37, showed disease ratings between 4 and 6 at 4 weeks after inoculation (Fig. 1C). As two virulence groups had already been described in *F. oxysporum* f. sp. *phaseoli* strains isolated from common bean plants (3), named highly virulent and weakly viru-

Fig. 1. Disease rates of Fusarium wilt induced in Spanish runner bean (*Phaseolus coccineus*) cultivar Judi3n de El Barco inoculated with different *Fusarium oxysporum* f. sp. *phaseoli* strains isolated from runner bean wilted plants: **A**, nine super virulent strains, **B**, 10 highly virulent strains, and **C**, seven weakly virulent strains. **D**, Disease rates of Fusarium wilt induced in *P. vulgaris* 'Blanca-Ri3n3n' inoculated with representative *F. oxysporum* f. sp. *phaseoli* strains isolated from runner bean wilted plants and two strains isolated from common bean wilted plants (FOP-SP01 and FOP-SP04).

lent, we also used these names for the second and third virulence groups found in the assays in the runner bean plants, and named as super virulent the first group, which is the most aggressive one.

The analysis of variance (ANOVA) using the F test of the disease ratings 4 weeks after inoculation confirmed the statistical significance of the suggested groups (at the 95% confidence interval). There are significant differences between super virulent and highly virulent strains ($P = 0.018$), and also between highly virulent and weakly virulent strains ($P = 0.016$), but there were not significant differences among strains within the same group of virulence: super virulent ($P = 0.253$), highly virulent ($P = 0.143$), and weakly virulent ($P = 0.209$).

As it may be observed in Figure 1, not only are the final disease ratings different for the three virulence groups, but also the progression of the disease is slower for the highly virulent group with respect to the super virulent, and the weakly virulent group with respect to the other two. This parameter can be conveniently measured by means of the DPR (17). DPRs showed by the super virulent strains are above 8.3, while the DPRs for the highly virulent strains are between 6.4 and 7.9 and those for the weakly virulent strains are between 3.1 and 6.0 (Table 1). Significance of DPR groups was confirmed by ANOVA ($P < 0.001$).

The response of Spanish common bean cultivar Blanca-Riñón to representative strains of the virulence groups isolated from runner bean indicates that all the tested strains induced severe symptoms in common bean and no differences in virulence could be observed (Fig. 1D). The disease ratings reached 4 weeks after inoculation were mostly above 8, the same range reached by the highly virulent strains originally isolated from common bean (FOP-SP01), and significantly higher than the disease ratings reached by the weakly virulent strain originally isolated from common bean and included as a control (FOP-SP04). The DPRs shown in Table 1 confirm these results, demonstrating that all the strains isolated from runner bean behave as highly virulent when inoculated in common bean, and clearly showing that virulence is host-specific and is not a characteristic of the pathogenic strain.

Fig. 2. Electrophoretic karyotypes of super virulent *Fusarium oxysporum* f. sp. *phaseoli* strains and highly virulent FOP-SP01 strain separated by pulsed-field electrophoresis and visualized by ethidium bromide staining. Arrows indicate chromosomal polymorphisms. Molecular sizes markers are chromosomes from *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*.

Accordingly with these results, we propose to name the three virulence groups described after the reactions induced in *P. coccineus* as Pc-super virulent (Pc-SP), Pc-highly virulent (Pc-HV), and Pc-weakly virulent (Pc-WV), and rename the formerly reported virulence groups described after the reactions induced in *P. vulgaris* as Pv-highly virulent (Pv-HV) and Pv-weakly virulent (Pv-WV).

VCGs. The analysis of VCGs was performed by means of complementation assays between mutants unable to use nitrate as the sole nitrogen source (*nit*). Therefore, different *nit* mutants (*nit1* and *nitM*) were recovered from all the analyzed strains. Vegetative compatibility analysis showed that none of the recovered mutants were compatible with representative mutants of the eight VCGs previously described in *F. oxysporum* f. sp. *phaseoli* (2,51). Four new VCGs were found among the 25 *F. oxysporum* f. sp. *phaseoli* isolates analyzed. VCG 0169 includes 21 isolates (FOP-SP13 to FOP-SP32), VCG 01610 includes two isolates (FOP-SP34 and FOP-SP35), and VCG 01611 and VCG 01612 include a single strain each, FOP-SP36 and FOP-SP37, respectively (Table 1). None of the isolates tested were self-incompatible (14).

IGS haplotypes diversity. Two types of polymorphisms were found in the IGS region of the ribosomal DNA among the 25 strains examined, both of which have already been described (2). First, the sizes of the fragments amplified were 2.55 kb, corresponding to the IGS-A haplotype, and 2.60 kb, corresponding to the IGS-B haplotype. A second level of polymorphism could be identified following analysis of restriction enzyme fragments. Each strain showed only one IGS fragment after PCR amplification. PCR products of the same size showed the same restriction fragment length polymorphism (RFLP) patterns, which were consistent with the size of the PCR products. Therefore, no IGS variation, in terms of size or restriction pattern, was detected within any of the strains tested, and each combination of size and RFLP pattern can be considered as an IGS haplotype.

The IGS-B haplotype identified corresponds to IGS-B2 according to the RFLP pattern of the amplicon (data not shown). The IGS-A haplotype was found to be the most abundant one, as it is present in 21 out of 25 strains (Table 1). However, the other four pathogenic strains tested had IGS-B2, which had been formerly found only in nonpathogenic Spanish strains and in a Brazilian pathogenic strain (ATCC 42145) (2).

Although most of the strains correspond to VCG 0169 and exhibit IGS-A haplotype, there is no absolute correlation between VCG and IGS haplotype, as strain FOP-SP33 was included in VCG 0169 and had IGS-B2 haplotype and strain FOP-SP34 was included in VCG 10610 and had IGS-A haplotype. Also, strains FOP-SP35, FOP-SP36, and FOP-SP37 showed IGS-B2 haplotype and corresponded to different VCGs.

MAT idiomorph. Genomic DNA from all the strains analyzed was obtained and used as template in PCR reactions with different oligonucleotide combinations as primers for the amplification of the two *F. oxysporum* MAT idiomorphs. The two amplicons obtained from the 25 strains were the 0.2-kb fragment obtained with primers FoMAT2P10/FMATp2, which corresponds to the 3' end of the MAT1-2 idiomorph, and the 0.6-kb fragment obtained with primers FMATp1/FoMAT2p2, which corresponds to the HMG motif of the MAT1-2 idiomorph (data not shown). These results show conclusively that all the strains analyzed exhibit MAT1-2 as mating type (Table 1).

EK patterns of super virulent strains. All the new strains classified as Pc-SV are included in VCG 0169 and show IGS-A haplotype and MAT1-2 idiomorph. EKs were obtained to rule out the possibility that they were clones of the same organism. In a former study we found small differences in EK patterns even among strains included in the same VCG, indicating that chromosomal patterns are highly variable in *F. oxysporum* f. sp. *phaseoli* (2).

The EK patterns observed were similar to the one obtained from the previously characterized strain FOP-SP01 (Fig. 2). How-

ever, some small differences could be detected. Strains FOP-SP13, FOP-SP15, FOP-SP16, and FOP-SP21 had seven bands of approximately the same sizes. The intensity of some of them indicated that there were groups of chromosomes (bands between 3.1 and 2.7 Mb and at around 5.7 Mb). FOP-SP14 showed also seven bands, but its second smaller chromosome (approximately 2.6 Mb) is slightly bigger than the equivalent one in the former group. FOP-SP17 and FOP-SP18 had eight bands, because of an extra chromosome at around 4 Mb. Finally, FOP-SP19 and FOP-SP20 also showed eight bands because of the existence of an extra band of approximately 0.8 Mb, but both strains could be differentiated because the band at approximately 3.1 Mb was slightly different in size. Therefore, five different EK patterns were obtained for the nine strains analyzed, although they were similar to that exhibited by the representative Pv-HV strain FOP-SP01.

Genetic diversity and virulence groups. As presented in Table 1, the Pc-SV strains share VCG, IGS haplotype, and MAT idiomorph, although they are not clonal because of some differences in the EK patterns (Fig. 2). Most of the Pc-HV strains belong to the same VCG (0169) and IGS-A haplotype with the exception of FOP-SP33, which shows IGS-B2 haplotype, and also share MAT idiomorph. The Pc-WV strains are distributed among all the VCGs (0169, 01610, 01611, and 01612). In fact, the VCGs 01610, 01611, and 01612 only include Pc-WV strains. Three of the Pc-WV strains have the IGS-B2 haplotype while four have the IGS-A haplotype. VCG 01610 is composed of only two Pc-WV strains that show different IGS haplotypes. Remarkably, VCG 0169 includes strains that have the same IGS haplotype but belong to the three virulence groups.

Detection of the gene *ftf1*. The main difference found so far, at the molecular lever, between Pv-HV and Pv-WV strains is the presence of multiple copies of the gene *ftf1* in the genome of Pv-HV strains (4). The multiple polymorphic copies of the gene *ftf1* were identified in *F. oxysporum* f. sp. *phaseoli* by means of Southern hybridizations of *Hind*III digested genomic DNA using probes B310-A280 and M18B-M19A, which correspond to fragments of the promoter and the coding region, respectively (42). In a similar way (Fig. 3), we have determined the number of copies of *ftf1* by Southern blot analysis of *Hind*III-digested DNAs of a number of strains isolated from runner bean and representative of the different virulence groups and VCGs. A representative strain

of the formerly described Pv-HP strains (FOP-SP01), Pv-WV strains (FOP-SP04), and a nonpathogenic strain (AB7) (2,3) were also included for comparative purposes.

The hybridization of genomic DNA with the probe M18B-M19A showed a pattern of five bands in most of the strains analyzed, which is identical to the pattern previously found in Pv-HV strains (FOP-SP01). One of these hybridizing fragments was also detected in *Hind*III-digested DNA from FOP-SP04 and AB7, suggesting cross-hybridization of the probe with a sequence partially similar to *ftf1*. In addition to the five common bands, three Pc-SV strains (FOP-SP13, FOP-SP17 and FOP-SP21), two Pc-HV strains (FOP-SP22 and FOP-SP29), and one Pc-WV strain (FOP-SP31) showed an extra hybridizing band (4.5 kb in size). On the other hand, two Pc-WV strains (FOP-SP36 and FOP-SP37) showed the same pattern as the Pv-WV and nonpathogenic strains.

Probe B310-A280 detected a number of polymorphic bands in *Hind*III-digested DNAs, which showed some correlation with virulence: five bands in Pc-SV strains, four to five for Pc-HV strains, and three to four for Pc-WV strains. A similar result was found when the same Southern blot was hybridized with probe B5-M17A, which corresponds to the coding region of the gene *ftf1* just downstream of probe B310-A280. Eleven hybridizing fragments, 8 to 11 hybridizing fragments, and 7 to 10 hybridizing fragments were detected in *Hind*III-digested DNA obtained from the Pc-SV strains, the Pc-HV strains, and the Pc-WV strains, respectively. However, this general correlation was not evident in some particular strains. For instance, strain FOP-SP31 is a Pc-WV strain and shows the same number of polymorphic bands as did the Pc-SV strains in Southern blot analysis using M18B-M19A as probe.

To get more insight into the number and architecture of the polymorphic copies of *ftf1* a BLAST search was performed against the genome of the reference strain *F. oxysporum* f. sp. *lycopersici* 4287, using the nucleotide sequence of the *ftf1a* copy isolated from *F. oxysporum* f. sp. *phaseoli* (GenBank accession No. DQ280313). BLAST queries using the coding region of *ftf1a* identified eleven homologous sequences in strain 4287 (Fig. 4), showing similarities between 97.8 and 84.6%. However, when the promoter regions of these copies were compared, only four sequences showed significant similarity. The analysis of the seven

Fig. 3. Southern hybridization of *Hind*III-digested genomic DNA from different strains of *Fusarium oxysporum*. Membranes were hybridized with different probes: M18B-M19A from the coding region of *ftf1*, which includes the fungal-specific transcription factor domain, B310-A280, which includes the promoter region of *ftf1*, and B5-M17A, from the coding region of *ftf1*, which includes the binuclear Zn(II)₂Cys₆ domain. The solid arrows and the dotted arrows show extra bands of some of the virulence groups; the dashed arrows show four bands common to all the strains in the hybridization with probe B5-M17A.

nonhomologous promoters indicated the presence of transposable elements (TEs) between 400 and 800 bp upstream of the common ATG codons (Fig. 4). One of the copies of *ftf1* (FOXG_09390) showed an 86-bp fragment homologous to the miniature inverted-repeat transposable element (MITE) named *mimp1* (21), while five copies of *ftf1* (FOXG_16414, FOXG_14000, FOXG_17084, FOXG_12539, and FOXG_12589) showed sequences homologous to the MITE *mimp4* (11). The seventh promoter (FOXG_17123) showed a region homologous to the first half (471 bp) of transposon *Impala* (11,21). We confirmed that the terminal inverted repeats (TIRs) of the found MITEs resembled those of *Impala*.

Comparison of the nucleotide sequences of the probes derived from *ftf1a* and the 11 copies of *ftf1* in the reference genome of strain 4287 confirmed that probe B310-A280 includes the less-conserved promoter region, where the TEs also appear; while probe B5-M17A includes the conserved binuclear zinc finger domain. A target for the restriction enzyme *Hind*III was found in 8 of the 11 copies located in the region between the probes B5-M17A and M18B-M19A. Additional targets for *Hind*III were found upstream and downstream of *ftf1* allowing the identification of homologous copies between *F. oxysporum* f. sp. *phaseoli* and *F. oxysporum* f. sp. *lycopersici* (data not shown).

Expression of the gene *ftf1*. The finding that some of the copies of *ftf1* found in *F. oxysporum* f. sp. *lycopersici* have TEs inserted in the promoter region, likely altering their function, could explain the lack of correlation between the number of copies of *ftf1* and the virulence displayed by some of the analyzed

strains. To test this hypothesis, the expression of gene *ftf1* was measured in planta by real-time RT-PCR 24 h after inoculation of runner bean plants with two representative strains of each of the three virulence groups described in this work. The strains were selected to represent the genetic diversity found in each virulence group (VCG 0169 and IGS-A in the SV strains, VCG0169 and IGS-A and IGS-B2 in the HV strains, and VCG 0169 and IGS-A, and VCG01610 and IGS-B2 in the WV strains). The expression of *ftf1* and the virulence degree, as measured by the DPR index, were compared for each strain analyzed (Fig. 5). Standard deviation of the relative expression of each strain is shown in Figure 5. When the uncertainty was estimated by evaluating the ratio using the $\Delta\Delta C_t$ s plus and minus of the standard deviation, the maximum and minimum relative expression values were 1.17 and 0.69 in Pc-SV strains, 0.70 and 0.44 in Pc-HV strains, and 0.42 and 0.11 in the Pc-WV strains.

A correlation between the expression of *ftf1* and the virulence displayed by each strain can be inferred from the results presented in Figure 5, as demonstrated by a Pearson correlation coefficient (r) of 0.97. When the error is considered by using the maximum and minimum (instead of the average) relative expression values of each strain, r is smaller but still significant ($r = 0.93$). A post-hoc power analysis with a confidence interval of 95% and sample size = 6 reveals a power over 0.9 in both cases (Power = 0.98 for $r = 0.97$ and 0.91 for $r = 0.93$).

Finally, to test whether the expression differences could be due to different ability to colonize the host, the relative amount of fungal/plant biomass was determined for each group of strains by

Fig. 4. Alignment of the copies of *ftf1* detected in *Fusarium oxysporum* f. sp. *lycopersici* strain 4287 genome (numbered from 01 to 11) and the *ftf1a* copy of *F. oxysporum* f. sp. *phaseoli*. The upper graph represents the similarity between all the aligned sequences. The black segments indicate similarity of the *F. oxysporum* f. sp. *lycopersici* copies of *ftf1* to the *ftf1a* copy. The light gray columns indicate the DNA fragments amplified in polymerase chain reactions using the indicated primers (black triangles), and used as probes in Southern hybridizations. The white triangles represent the putative start and stop codons. The white arrows indicate the DNA regions that show homology to transposable elements. The light gray arrows indicate the position of the Zn(II)₂Cys₆ binuclear zinc finger domain. The dark gray arrows indicate the position of a putative intron.

real-time PCR using pairs of primers corresponding to the *F. oxysporum* *EF1* gene and the *P. vulgaris* microsatellite BMd53. The results obtained in three separate experiments yielded 20 mg/g for Pc-SV strains, 60 mg/g for Pc-HV strains, and 3 mg/g for Pc-WV. Therefore, no correlation between expression and host colonization could be concluded.

DISCUSSION

Twenty-five *F. oxysporum* isolates were obtained from diseased runner bean (*P. coccineus*) plants showing symptoms of vascular wilt (Fusarium wilt) and analyzed for genetic diversity, virulence, and copy number and expression of the gene *ftf1*. The isolations were made from portions of stems and all the isolates were pathogenic when tested in inoculation assays both on runner bean and common bean (*P. vulgaris*) plants, thus confirming that they are *F. oxysporum* f. sp. *phaseoli* J.B. Kendrick and W.C. Snyder strains. This result is in contrast with previous work on the isolation of *F. oxysporum* strains from severely wilted common bean plants, which yielded a reduced fraction of pathogenic strains compared with nonpathogenic ones (2,15).

The *F. oxysporum* strains analyzed here were placed in four new VCGs (0169, 01610, 01611, and 01612), two IGS haplotypes (IGS-A and IGS-B2) previously described (2) and MAT1-2. Most of the strains are vegetatively compatible (VCG 0169) and exhibit IGS-A haplotype, which is also the haplotype shown by all the pathogenic strains isolated from common bean. However, the strain FOP-SP33 shows IGS-B2 haplotype and belongs to VCG 0169, and also the two strains included in VCG 01610 show different haplotypes. Previous studies had shown no variation in IGS haplotype and little variation in the EK patterns within each VCG, supporting the clonal origin of vegetatively compatible strains (2,26,27,32). Our results support this hypothesis, but the exceptions may indicate high mutation rates in certain loci, like those involved in vegetative compatibility, and certain genomic regions, like the ribosomal IGS. Small genetic differences between vegetative compatible strains have already been reported (10,38). Another striking finding relates to the pathogenic strains that show IGS-B2 haplotype. While the formerly characterized strains isolated from common bean plants in the area of El Barco de Avila displayed IGS-A haplotype (2), only some nonpathogenic strains and a Brazilian pathogenic strain (ATCC 42145) showed IGS-B2 haplotype. These results support a polyphyletic origin for at least some *formae speciales* (1,2,27,39). The mechanisms underlying the convergent evolution for host pathogenicity are still largely unknown. Recently, the horizontal gene transfer of lineage-specific chromosomes of *F. oxysporum* and the subsequent conversion of a nonpathogenic strain into a pathogen has been reported (33). If these kinds of events also occur in the field, they would explain why nonrelated lineages share host-specific pathogenicity genes and virulence factors.

The susceptibility reactions of *P. coccineus* 'Judi3n de El Barco' to the 25 characterized strains allowed their classification into three virulence groups. The nine Pc-SV strains are genetically very homogeneous, as they are vegetatively compatible (VCG 0169), share IGS-A haplotype, and MAT. Only minor differences in the EK patterns were found that distinguish some of them, indicating the plasticity of *F. oxysporum* karyotypes (2,25, 37). Eight of the nine Pc-HV strains also are vegetatively compatible (VCG 0169) and share IGS-A haplotype and MAT, while strain FOP-SP33 shows IGS-B2. The group of weakly virulent pathogens is more diverse as it includes seven strains that belong to the four VCGs, and show IGS-A and IGS-B2 haplotypes. It is worth noting that VCG 0169 includes strains that belong to the three virulence groups.

Inoculation assays developed on *P. vulgaris* 'Blanca-Ri3n' with representative strains of the three virulence groups showed that they all behaved as the previously described highly virulent

strains (Pv-HV) (3). The scarce studies previously conducted in *P. coccineus* and *P. vulgaris* cultivars inoculated with *F. oxysporum* f. sp. *phaseoli* strains only scored resistant versus susceptible reactions (1,44), which depend on the race structure of the pathogen. Our previous work showed that Spanish race 6 *F. oxysporum* f. sp. *phaseoli* strains could exhibit different degrees of virulence depending on the genetic background of the *P. vulgaris* cultivar inoculated (Central American or Andean origin). Therefore, virulence is host specific in a way that is not exclusively related to the race structure of a pathogen population or merely a pathogen characteristic. The results presented here are consistent with our former work and indicate that the degree of virulence displayed by a certain strain depends not only on the cultivar but also the host species, which strongly suggests that the degree of damage caused to the host is the result of a coevolution between certain virulence factors in the pathogen and genetic determinants in the host (either different cultivars or species). Coevolution between common bean cultivars and their pathogens has also been suggested for the interactions between this species and the pathogens *Phaeoisariopsis griseola* (20) and *Colletotrichum lindemuthianum* (18). However, in these studies no fungal genetic factor was found to be involved in such coevolution.

The only genetic difference found to date between the virulence groups is the presence of multiple copies of the gene encoding the transcription factor *ftf1* in Pv-HV but not Pv-WV strains (4,42). This gene is highly up-regulated during early stages of infection, and all the copies found to date in *F. oxysporum* f. sp. *phaseoli* are linked to transposon *Marsu* (42). The analysis of the number of copies of *ftf1* found in *F. oxysporum* f. sp. *phaseoli* strains isolated from *P. coccineus* did not show a clear correlation between the number of polymorphic copies and the degree of virulence. For example, the number of copies present in Pc-WV strains ranged from none (FOP-SP36 and FOP-SP37), like Pv-WV strains, to six (FOP-SP31), like Pc-SV strains. Recently, the nucleotide sequence of the *F. oxysporum* f. sp. *lycopersici* strain 4287 has been made available, showing the expansion of a gene family coding for transcription factors related to *ftf1* (33). In silico analysis of the genome of strain 4287 reveals the presence of

Fig. 5. Real-time reverse transcription-polymerase chain reaction analysis of the expression of *ftf1* in runner bean plants 24 h after inoculation with representative strains of *Fusarium oxysporum* f. sp. *phaseoli*. An arbitrary value of 1.0 was denoted for the transcript level of *ftf1* 24 h after inoculation with strain FOP-SP13. Gray bars represent the relative expression of *ftf1* as measured in plants inoculated with the different strains. Light bars represent the degree of virulence displayed by the same strains as measured by the disease progression rate index. Standard deviations of the relative expression of each strain are indicated by vertical lines.

eleven polymorphic copies of *ftf1* and, by comparing *Hind*III sites, we have been able to identify homologous copies in the *formae speciales phaseoli* and *lycopersici*. Seven copies of *ftf1* in *F. oxysporum* f. sp. *lycopersici* show insertions in the promoter region homologous to transposable elements *mimp1*, *mimp4*, and *Impala*. These insertions most likely either inactivate the function of *ftf1* or alter its expression, and would explain the lack of an absolute correlation between the number of copies of *ftf1* and virulence.

The analysis of virulence displayed by the most genetically homogeneous strains (those sharing VCG 0169, IGS-A, and MAT1-2) shows a range from weakly virulent to super virulent strains. Among the two weakly virulent strains, FOP-SP31 and FOP-SP32, the latter shows four polymorphic copies of *ftf1* when using as probe an internal fragment of the coding region (M18B-M19A), while the other shows five polymorphic copies. However, the hybridization patterns when using as probes fragments of the promoter (B310-A280) or the 5'-end of the coding region (B5-M17A) show less polymorphic copies in these two weakly virulent strains than in the super virulent or highly virulent strains, thus confirming alterations in the promoter and the beginning of the coding region. These results suggest that, depending on the strain, a variable number of the polymorphic copies of *ftf1* may be functionally altered. If this is true, then the number of polymorphic copies and virulence would show a rough correlation, while a more precise correlation would be indicated by the level of expression of *ftf1* in infected plants. An alternative explanation is that the observed differences in virulence could be due to a different ability to colonize the host, and then the expression differences would be the result of differences in virulence, and not the cause. In planta measurements of *ftf1* transcripts, as quantified by real-time PCR, demonstrate a correlation between relative expression of *ftf1* and virulence. The range of *ftf1* expression in the strains representative of the different virulence groups do not overlap, supporting the biological significance of this correlation. At the time of the maximum expression of *ftf1* (12 to 24 h), the differences observed in host colonization could not be correlated with the virulence groups. Therefore, the ability to colonize the host may be one of the causes that explain virulence, but not the only one, and it should be concluded that expression of *ftf1* is another cause of virulence, and not an effect, in *F. oxysporum*.

Here we have shown that the virulence phenotype is the result of interactions between genetic factors both in the host and the pathogen. The analysis of the genetic factors involved in the determination of virulence may be quite difficult if the effects of different ones superimpose in the final phenotype. Clonal populations of pathogens, as several strains analyzed in this work, offer a good opportunity to correlate phenotypic variation with single molecular differences. In this work we have shown that the level of *ftf1* transcript account for the virulence phenotypes observed in the interaction of a genetically homogeneous population of *F. oxysporum* f. sp. *phaseoli* isolates with *P. coccineus* plants growth in a reduced area. The most similar interaction reported to date is the one determined by the multiple copy locus *Tox*, responsible of the production of the host specific toxins (HST) *ToxB* in the wheat pathogen *Pyrenophora tritici-repentis* (28,34,35). It has been shown that quantitative variation in the virulence of *Pyrenophora tritici-repentis* and the development of appressoria is related to the extent of *ToxB* gene expression (5). In *F. oxysporum* a number of genes have been identified whose inactivation results in loss of virulence (reviewed in 36), but most of them are single genes involved in specific functions or members of gene families. Among the transcription factors analyzed to date, *FNRI* (16), *CTI6* (36), *PacC* (13), and *fst12* (17,46) have roles in virulence. Inactivation of these genes normally results in reduced virulence, except the inactivation of the gene *PacC* that induces an increase in virulence and the expression of acid-induced genes (13).

None of the above mentioned virulence factors of *F. oxysporum* are present as multiple copy genes or are linked to transposons as is the case of *ftf1*. However, the relationship with transposons is a regular feature in several genes related to pathogenicity. In *F. oxysporum* f. sp. *lycopersici* gene *Six1-H*, a truncated copy of the avirulence gene *Six1*, has its coding region interrupted by transposon *Drifter* (43). The insertion of a transposon in the promoter of the gene *Avr-Pita* of *Magnaporthe oryzae* causes the loss of the gene function (22). Some of the regions proximal to the multiple copies of gene *ToxB* of *Pyrenophora tritici-repentis* have transposons fragments (34). Finally, some of the virulence genes found in *F. solani* (*Nectria haematococca*) are located in a small supernumerary chromosome showing a great density of transposons (30,47). Interestingly, it has been found that almost 47% of the identified MITEs in the *F. oxysporum* f. sp. *lycopersici* genome are located in chromosome 14 (11), one of the smallest chromosomes of *F. oxysporum* f. sp. *lycopersici* and one of the four chromosomes entirely composed of lineage-specific (LS) sequences (33). In *F. oxysporum* f. sp. *phaseoli* the multiple copies of *ftf1* are linked to the only copies of transposon *Marsu* found in this *forma specialis* and located in the smallest chromosome of the Pv-HV strains (42). The genes present in the *F. oxysporum* f. sp. *lycopersici* LS regions are significantly enriched for secreted effectors and virulence factors, transcription factors, and protein involved in signal transduction (33). The gene *ftf1* may be easily classified into these three categories and the accumulated evidence shows that it is a characteristic LS sequence in *F. oxysporum*. Our results highlight a role for *ftf1* in the modulation of the virulence response and possibly its coevolution with the interacting factors in the hosts' genomes, although more experimental evidence is needed to demonstrate the causal link between *ftf1* and virulence. Gene silencing of *ftf1* and expression of *ftf1* in weakly virulent strains are currently underway in our lab to shed light on this important question.

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