

Effector Gene Suites in Some Soil Isolates of *Fusarium oxysporum* Are Not Sufficient Predictors of Vascular Wilt in Tomato

Nicolas A. Jelinski, Karen Broz, Wilfried Jonkers, Li-Jun Ma, and H. Corby Kistler

First author: Department of Soil, Water and Climate, University of Minnesota-Twin Cities, 1991 Upper Buford Circle, Saint Paul 55108; second and fifth authors: U.S. Department of Agriculture-Agricultural Research Service, Cereal Disease Laboratory, 1551 Lindig Street, University of Minnesota-Twin Cities, Saint Paul 55108; third author: Bejo Zaden BV, Trambaan 1, 1749CZ Warmenhuizen, The Netherlands; and fourth author: Department of Biochemistry and Molecular Biology, University of Massachusetts-Amherst, Amherst 01003.

Accepted for publication 13 March 2017.

ABSTRACT

Seventy-four *Fusarium oxysporum* soil isolates were assayed for known effector genes present in an *F. oxysporum* f. sp. *lycopersici* race 3 tomato wilt strain (FOL MN-25) obtained from the same fields in Manatee County, Florida. Based on the presence or absence of these genes, four haplotypes were defined, two of which represented 96% of the surveyed isolates. These two most common effector haplotypes contained either all or none of the assayed race 3 effector genes. We hypothesized that soil isolates with all surveyed effector genes, similar to FOL MN-25, would be pathogenic toward tomato, whereas isolates lacking all effectors would be nonpathogenic. However,

inoculation experiments revealed that presence of the effector genes alone was not sufficient to ensure pathogenicity on tomato. Interestingly, a nonpathogenic isolate containing the full suite of unmutated effector genes (FOS 4-4) appears to have undergone a chromosomal rearrangement yet remains vegetatively compatible with FOL MN-25. These observations confirm the highly dynamic nature of the *F. oxysporum* genome and support the conclusion that pathogenesis among free-living populations of *F. oxysporum* is a complex process. Therefore, the presence of effector genes alone may not be an accurate predictor of pathogenicity among soil isolates of *F. oxysporum*.

Fusarium oxysporum is a free-living fungus that is ubiquitous in soils worldwide, is involved in the cycling of heavy metals and nitrogen, and can be pathogenic toward organisms as diverse as tomato, cotton, banana, and humans (Corrales Escobosa et al. 2010; Kistler 2001; Long et al. 2013; Ma et al. 2013). The *F. oxysporum* species complex also includes numerous nonpathogenic strains, some of which have been shown to be effective in plant protection or biological control (Alabouvette et al. 2009).

The *F. oxysporum* species complex has been divided among vegetative compatibility groups (VCGs) based on the ability of isolates to fuse and produce stable heterokaryotic cells (Elias and Schneider 1991); such compatibility only occurs among very closely related strains. In addition to vegetative compatibility, two idiomorphic alleles of a single mating-type locus (*MAT1*) are recognized in *F. oxysporum* (Arie et al. 2000) and have been designated *MAT-1* (*MAT-1-1*) and *MAT-2* (*MAT-1-2*), although the role they play in controlling mating or genetic variation in the species is a matter of debate as there is no known sexual stage (Irzykowska and Kosiada 2011; Lievens et al. 2009b).

Within the *F. oxysporum* species complex, *formae speciales* (f. sp.) have been defined as groups of strains with shared host specificity (Armstrong and Armstrong 1981). *Formae speciales* have been shown to be polyphyletic (O'Donnell et al. 1998), and most *formae speciales* consist of more than one VCG, with each VCG corresponding to a particular clonally derived lineage (Attitalla et al. 2004; Kistler 2001; Klein and Correll 2001). *F. oxysporum* f. sp. *lycopersici* is an economically important and globally distributed wilt pathogen of tomato (Armstrong and Armstrong 1981) and *F. oxysporum* f. sp. *lycopersici* strains appear to have horizontally

transferred accessory chromosomes (Ma 2014; Ma et al. 2010) which encode a suite of putative effectors—including a suite of effectors termed SIX proteins because they are Secreted In Xylem fluids (Houterman et al. 2009). These observations are not restricted to *F. oxysporum* f. sp. *lycopersici*. Recent comparative analyses across a range of host specificities has shown that strains in the same *forma specialis* from different VCGs carry identical effector genes (van Dam et al. 2016).

Several SIX genes are associated with the three presently described races of *F. oxysporum* f. sp. *lycopersici* (Gale et al. 2003; Houterman et al. 2009). Single-gene resistance (I) in tomato to race 1 pathogens was eventually overcome by the loss of *SIX4* (corresponding to AVR1), leading to race 2 strains (Houterman et al. 2009). Race 3 strains have a probable center of origin in commercial tomato fields in Florida and evade a second major tomato resistance gene (*I-2*) by a single amino acid change in AVR2 (*SIX3*) (Gale et al. 2003; Houterman et al. 2009). Therefore, race 1 pathogens contain all known SIX genes, while race 2 and 3 pathogens do not contain *SIX4* and race 3 strains have a single amino acid change in *SIX3* relative to race 2 (Houterman et al. 2009).

Currently, genetic markers for 12 SIX genes have been explored in the published literature: *SIX1* to *SIX11* and *SIX14* (Chakrabarti et al. 2010; Laurence et al. 2015; Meldrum et al. 2012; Schmidt et al. 2013). A global survey of several SIX genes (*SIX1* to *SIX7*) confirmed that these horizontally transferred effector genes (Ma et al. 2010; Shahi et al. 2016) are a robust method for identification of host specificity in *F. oxysporum* strains isolated directly from plant tissues (Lievens et al. 2009a). The presence of *SIX1*, *SIX2*, *SIX3*, and *SIX5* were found to be unambiguous in their identification of *F. oxysporum* f. sp. *lycopersici* in isolates obtained from plant tissues (Lievens et al. 2009a).

In vitro studies have shown that the horizontal transfer of accessory chromosomes (with their corresponding suite of effectors, including SIX genes) may occur even among vegetatively incompatible strains under the right environmental conditions (Ma et al. 2010; Shahi et al. 2016). We note that in this context, vegetative compatibility is based on visual indications of heterokaryon

Corresponding author: N. A. Jelinski; E-mail address: jeli0026@umn.edu

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2017.

formation, which will only be apparent if the number of fusions is sufficient to provide the metabolites required for wild type growth. Therefore, stains that appear to be incompatible by this test may nevertheless undergo anastomosis to some extent. Nonetheless, the frequency, efficiency, and effectiveness of these exchanges in the environment (particularly in free-living soil populations of *F. oxysporum*), as well as the factors that might influence those dynamics such as vegetative compatibility and mating type, remain largely unknown.

Limited evidence from recent studies suggests that the frequency and abundance of SIX genes in soil isolates from natural ecosystems is low. Rocha et al. (2016) examined the presence or absence of 12 SIX genes across 115 *F. oxysporum* soil isolates in a continental wide survey across Australia and found low levels of SIX gene presence (<6% of all isolates [SIX9], <2% of all isolates [SIX1, SIX6, and SIX8]). In a study of 433 *F. oxysporum* isolates collected from wild *Solanum* spp. tissues and rhizosphere soil in the Peruvian Andes, Inami et al. (2014) found no strains which carried assayed SIX genes and none which caused vascular wilt on susceptible tomato plants.

Based on the body of previous work, we developed three hypotheses. First, we hypothesized that soil isolates from tomato fields undergoing a wilt epidemic would have a higher prevalence of SIX genes than those from natural ecosystems. Second, we hypothesized that soil isolates of *F. oxysporum* in tomato fields under wilt pressure would have either (i) no SIX genes or (ii) the full complement of *F. oxysporum* f. sp. *lycopersici*-specific SIX genes (SIX1, SIX2, SIX3, and SIX5), as might be expected for an asexual fungus with known capacity for horizontal transfer of pathogenicity-associated chromosomes. Finally, we hypothesized that any soil isolates that contained all surveyed SIX genes would be pathogenic toward tomato, while those that did not would not demonstrate pathogenicity toward tomato.

To test these hypotheses, we surveyed effector gene variability (assessed by presence or absence of PCR markers for seven SIX genes) among a collection of 74 free-living *F. oxysporum* isolates from soil material between rows and at the base of tomato plants in Manatee County, Florida. When SIX gene variability between these soil isolates was detected, we conducted pathogenicity tests on a subset of these isolates representing various effector haplotypes to understand the connection between variability in effector gene suites and pathogenic action against tomato. Lastly, when we discovered discrepancies between hypothesized pathogenic action and SIX genes, we developed potential explanations for these observations through electrophoretic karyotyping, vegetative compatibility, and sequencing of SIX genes.

MATERIALS AND METHODS

Fungal isolates. Soil cores (5 cm depth) were taken in July 1996 from two tomato production fields with symptoms of vascular wilt in Manatee County, FL where *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici* were both present and from which the sequenced race 3 pathogenic isolate MN-25 was first isolated (Gale et al. 2003; Ma et al. 2010). Ten soil samples each were taken in (i) two different interrow locations in field 1 (termed locations 1 and 2) and (ii) a row location (within 5 cm of the base of individual tomato plants) and an interrow location in field 2 (termed locations 3 and 4, respectively). Within 48 h of sampling, and without air drying, 1 ml of diluted soil suspension (1×10^{-3} g of soil/ml of sterile water) for each soil sample was inoculated onto plates of Komada's *Fusarium* medium (Komada 1975; Leslie and Summerell 2006). Growth on this medium results in the selective growth and pigmentation of *F. oxysporum* colonies, which can be confirmed by their distinctive conidial morphology (Garibaldi et al. 2004). The inoculated plates were incubated at 25°C for 5 days under 12 h periods of alternating light and darkness. Repeated culturing efforts were performed for soil suspensions failing to produce more than five *Fusarium* isolates after the initial plating.

Individual randomized colonies of *Fusarium* were subcultured onto half-strength potato dextrose agar (1/2-PDA) and grown under a 12-h photoperiod at 25°C for 5 to 7 days. These individual colonies were subsequently cataloged, grown in liquid culture to promote sporulation, and filtered through Miracloth to isolate microconidia which were prepared for long-term storage in 50% glycerol stock at -70°C. These efforts resulted in a catalog of 148 individual single-spored isolates of *F. oxysporum*.

Seventy-four *F. oxysporum* isolates from the full collection of 148 isolates were randomly selected for further study in 2011: 36 from the two interrow locations in field 1 (19 isolates from interrow location 1 [1-IR] and 17 isolates from interrow location 2 [2-IR]) and 38 isolates from field two (12 from row location 3 [3-R] and 26 isolates from interrow location 4 [4-IR]). The differences in selected isolate numbers from each location reflect the differing total numbers of isolates from each location in the full collection. Half of all isolates in each location were randomly selected. These individual isolate accessions were named with the generic acronym FOS (*F. oxysporum* soil), followed by a number representing the collection location (1, 2, 3, or 4, described above) and a unique numerical identifier (i.e., FOS 1-3). Additionally, four previously sequenced strains of *F. oxysporum* were used as positive or negative controls: FOL 4287 (f. sp. *lycopersici*, NRRL 34936), MN-25 (f. sp. *lycopersici*, NRRL 54003), CL57 (f. sp. *radicis-lycopersici*, NRRL 26381) and FO47 (biocontrol strain, NRRL 54002) (Broad Institute of Harvard and MIT at <https://www.broadinstitute.org/>) (Ma et al. 2010). FOL 4287 is a well-characterized strain of *F. oxysporum* f. sp. *lycopersici* (race 2, VCG 0030) isolated from infected tomato. MN-25 (NRRL 54003) is a strain of *F. oxysporum* f. sp. *lycopersici* (race 3, VCG 0033) isolated from diseased tomato plants on the same fields as the soil isolates in this study at a farm in Manatee County, Florida (Gale et al. 2003). CL57 (NRRL 26381) is a strain of tomato crown rot pathogen *F. oxysporum* f. sp. *radicis-lycopersici* (VCG 0094) from Collier County, Florida (Rosewich et al. 1999). The strain FO47 is a nonpathogenic strain that colonizes plant roots and shows biological control properties by suppressing wilt diseases (Fravel et al. 2003).

DNA isolation and PCR conditions. Microconidia from long-term (-70°C) glycerol storage stocks were streaked onto PDA plates and cultured for 5 to 7 days at 21°C. Mycelium was scraped off and transferred to petri dishes (15 cm diameter) containing approximately 70 ml of PDB. After 7 days of growth in still culture at room temperature, the resulting mycelium was harvested by filtration through cheesecloth, rinsed with sterile deionized water, blotted dry, and placed into 15-ml polypropylene tubes. The mycelium was kept frozen at -70°C and lyophilized for at least 48 h before DNA extraction.

Up to 0.2 g of freeze-dried mycelium was pulverized and 7 ml of DNA extraction buffer was added. The tubes were vortexed for 1 min and kept in a 65°C waterbath for 60 min followed by a chloroform extraction. To the aqueous phase, 200 µl of NaCl-cetyltrimethylammonium bromide (CTAB) (10% CTAB and 0.7 M NaCl) and 2 ml of CTAB precipitation buffer (1% CTAB; 50 mM Tris, pH 8; and 10 mM EDTA, pH 8) were added. Tubes were shaken and incubated at room temperature for 20 min before centrifugation. The DNA pellet was resuspended in 7 ml of NaCl-Tris-EDTA (TE) buffer (1 M NaCl; 10 mM Tris, pH 8; and 1 mM EDTA, pH 8), followed by a second chloroform extraction. The DNA in the aqueous phase was precipitated by adding 7 ml of isopropanol and then pelleted at $14,000 \times g$ for 20 min at 21°C. The DNA was resuspended in 400 µl of TE (10 mM Tris and 1 mM EDTA, pH 8.0), transferred to a microfuge tube, precipitated, and centrifuged. The DNA pellet was resuspended in 50 to 100 µl of TE and allowed to dissolve at 4°C for several days before DNA concentration was determined using a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco).

Genomic DNA was quantified and diluted to 10 ng µl⁻¹, and 10 ng of total DNA was used in each 20-µl PCR reaction along with

10 pmol of each primer (forward and reverse), 0.5 units of Taq, 0.2 mM each dNTPs, and 4 µl of Takara 10× buffer (a total of 4 mM MgCl₂). Previously published primers for effectors SIX1 to SIX7 (Lievens et al. 2009a; van der Does et al. 2008), FEM1—a conserved cell wall glycoprotein used as a positive control (Jonkers and Rep 2009), *F. oxysporum* mating-type idiomorphs MAT1-1 and MAT 1-2 (Arie et al. 2000), and an *F. oxysporum*-specific portion of translation elongation factor 1-α gene *Fef* (Haegi et al. 2013) were utilized (Table 1). We chose to assess markers of SIX1 to SIX7 only (and not SIX8 to SIX12) because of the foundational published work that has been completed for these markers, as well as indications that the presence or absence of these genes are able to unambiguously identify *F. oxysporum* f. sp. *lycopersici* in a global survey of *F. oxysporum* plant isolates (Lievens et al. 2009a).

PCR conditions were as follows: for SIX1 to SIX7, FEM1, and *Fef*: 94°C for 2 min; 32 cycles of 94°C denaturation for 45 s, 59°C annealing for 45 s; 72°C for 45 s; and a final extension at 72°C for 10 min; for MAT1-1 and MAT 1-2: 94°C for 2 min; 30 cycles of 94°C denaturation for 30 s, 58°C annealing for 30 s; 72°C for 45 s; and a final extension at 72°C for 6 min. Amplicons were resolved in 1.5% agarose gels stained with 0.5 µg ml⁻¹ of ethidium bromide and standardized with Takara 1-kb DNA ladder. Isolates were scored for presence/absence of amplicons and haplotype codes were assigned to isolates based on their SIX gene complement, as indicated by PCR assays. Isolates with no detected amplicons were labeled SIX₀, while isolates with detected amplicons were labeled according to the SIX gene amplicon suite (i.e., an isolate which was positive for amplicons for SIX₂ and SIX₃ would be labeled SIX₂₃).

Pathogenicity tests and inoculations. A subset of the initial 74 isolates representative of four observed effector haplotypes (eight isolates: FOS 2-17, FOS 3-1, FOS 3-15, FOS 4-9, FOS 4-19, FOS 1-23, FOS 4-4, and FOS 3-5) was selected for pathogenicity testing, alongside three control strains (FO47, FOL 4287, and FOL MN-25). Microconidia (spore) suspensions from each isolate were prepared by shaking plugs from actively growing PDA culture in liquid KNO₃ medium (Leslie and Summerell 2006) for 4 days at 150 rpm/25°C. All spore suspensions were adjusted to 1 × 10⁷ spores ml⁻¹. Fourteen-day-old seedlings of a fully susceptible heirloom tomato cultivar (Moneymaker) were inoculated by the root-dip method. Briefly, seedlings were carefully separated from vermiculite, root tips were cut with a sterile surgical blade and dipped into spore suspension for 5 min. Seedlings were then planted in pots with potting soil/peat mix and grown with daily cycles of 12 h of light and 12 h of darkness at 24°C. Four completely

independent rounds of pathogenicity tests were conducted, resulting in 60 to 75 scored plants for every tested isolate.

Plants were scored after 21 days (Michielse et al. 2009) based upon visual inspection of stem cross-sections with a 5× hand lens, cut just above cotyledons (0 = plant without symptoms, 1 = one to two brown vessels, more than four true leaves, 2 = three or more brown vessels, more than four true leaves, and 3 = three or more brown vessels, four or less true leaves). This scoring scheme is similar to that proposed by De Cal et al. (1995) but does not explicitly include assessments of leaf yellowing in order to avoid false positives due to the large number of potential nonpathogens included in the study. All plants scoring a 1 or greater also showed evidence of leaf yellowing in conjunction with brown xylem vessels. The weight of plant biomass above the cotyledons (as cut for visual inspection of xylem vessels) was recorded at the time of scoring.

Electrophoretic karyotyping (CHEF) and Southern hybridization. Electrophoretic karyotyping by contour-clamped homogeneous electric field (CHEF) electrophoresis was performed as described previously (Ma et al. 2010, Jonkers et al. 2014). Plugs containing 4 × 10⁸/ml protoplasts were loaded on a CHEF gel (1% Fastlane agarose [FMC] in 0.5× TBE) and run for 255 h, using switch times between 1,200 to 4,800 s at 1.8 V/cm (=60 Volt). Chromosomes of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* were used as molecular size markers (Bio-Rad). DNA was depurinated in 0.25M HCl and transferred from the CHEF gel to HyBond XL (GE Health Care) membrane under standard alkaline conditions according to the manufacturer's protocol. The membrane containing transferred DNA was rinsed in 2× SSC, air-dried, and baked at 80°C. A nonradioactive Southern protocol was used to probe for products of interest. Probes for SIX3 and SIX6 were amplified by PCR conditions described above using primers SIX3-F1/SIX3-R1 and SIX6-F1/SIX6-R1 (Table 1) and purified using the Qiaquick Gel Extraction kit (Qiagen). Probe hybridization and detection was performed using an AlkPhos Direct labeling kit with CDP-Star chemiluminescent detection reagent (GE Health Care) according to the manufacturer's protocol. Images were obtained using the Carestream Image Station 4000MM Pro.

Nitrate nonutilizing (nit) mutant generation and vegetative compatibility testing. Plugs from actively growing isolates on Czapek-Dox agar (CDA) culture were grown on 3, 4, and 5% chlorate media (Leslie and Summerell 2006) until resistant sectors appeared (~2 weeks). These putative nit mutants were subsequently phenotyped by comparing their growth on CDA, NO₂,

TABLE 1. Primers used for polymerase chain reactions and DNA sequencing in this study

Code	Sequence 5'-3'	Target	Amplicon length (bp)	Reference
P12-F2B	GTATCCCTCCGATTGTTGAGC	SIX1	992	van der Does et al. 2008
P12-R1	AATAGAGCCTGCAAGCATG	SIX1		Rep et al. 2005
SIX2-F2	CAACGCCGTTTGAATAAGCA	SIX2	749	van der Does et al. 2008
SIX2-R2	TCTATCCGCTTTCTTCTCTC	SIX2		van der Does et al. 2008
SIX3-F1	CCAGCCAGAAGGCCAGTTT	SIX3	608	van der Does et al. 2008
SIX3-R2	GGCAATTAACCACTCTGCC	SIX3		van der Does et al. 2008
SIX4-F1	TCAGGCTTCACTTAGCATAC	SIX4	967	Lievens et al. 2009a
SIX4-R1	GCCGACCGAAAAACCCATA	SIX4		Lievens et al. 2009a
SIX5-F1	ACAGCTCTACTACTCTTCA	SIX5	667	Lievens et al. 2009a
SIX5-R1	GAAAACCTCAACGCGGCAAA	SIX5		Lievens et al. 2009a
SIX6-F1	CTCTCTGAACCATCAACTT	SIX6	793	Lievens et al. 2009a
SIX6-R1	CAAGACCAGGTGTAGGCATT	SIX6		Lievens et al. 2009a
SIX7-F1	CATCTTTTCGCCGACTTGGT	SIX7	862	Lievens et al. 2009a
SIX7-R1	CTTAGCACCTTGAGTAACT	SIX7		Lievens et al. 2009a
FEM1-F	AAAGAATTACCTCCGCCACTGGTGACTC	FEM1	240	Jonkers and Rep 2009
FEM1-R	AAATCTAGACCCTCTCAGGGACACTGG	FEM1		Jonkers and Rep 2009
MAT1-F	AGAGCTGCCGATGAAGTG	MAT1	834	Arie et al. 2000
MAT1-R	TGTTTCATCAAAGGGCAAGCG	MAT1		Arie et al. 2000
MAT2-F	ATACTGCATCTTAGAGTGAGG	MAT2	806	Arie et al. 2000
MAT2-R	GAAGACGATGGGGCTGGAA	MAT2		Arie et al. 2000
Fef1F	TAGTCACTTTCCTTCAATCGC	Ef1	142	Haegi et al. 2013
Fef2R	AAGTGGCGGGGTAAGT	Ef1		Haegi et al. 2013

and Hx media (Leslie 1990) identifying WT, nit1, nit3, and NitM mutations. NitM and nit1 mutants, which are biochemically complementary and produce clear growth patterns and unambiguous growth on all three media were the preferred pairing for VCG testing. Pairings of nit1 and nit3, which may also be biochemically complementary although more phenotypically ambiguous, were useful in confirming the results of initial VCG tests. Plugs from mutants of complementary groups from different isolates were grown starting 6 cm apart on opposite sides of a CDA plate for 7 to 14 days. Primary pairings were conducted between nit1 and NitM mutants, and secondary, confirmational pairings were between nit1 and nit3 mutants. Vegetative compatibility of isolates was assumed when a zone of dense, aerial growth emerged where the hyphae from each isolate met on minimal medium. Strains were considered not vegetatively compatible when hyphae grew past each other without producing a dense zone of growth, instead with minimal, expansive spreading hyphae (Leslie and Summerell 2006). All compatibility tests were run twice with the same pairs of mutants to confirm results.

Sanger sequencing. SIX gene amplicons (*SIX1*, *SIX2*, *SIX3*, *SIX5*, *SIX6*, and *SIX7*) from single bands (using the same PCR conditions as above but scaled for 50- μ l reactions) in seven isolates (FOL 4287, FOL MN-25, FOS 2-9, FOS 2-11, FOS 2-17, FOS 4-4, and FOS 3-5) were sequenced by single pass using both forward and reverse primers (Table 1). Sequences were quality controlled using 4Peaks software (Griekspoor and Groothuis 2005) and aligned with Clustal Omega (Sievers et al. 2011). Sequences obtained for the assayed effector genes in this study were deposited in GenBank under accession numbers KM047024 to KM047041.

RESULTS

Survey of *F. oxysporum* soil isolates for SIX, MAT, and species specific loci. The results of PCR assays for SIX genes and MAT idiomorph markers are shown in Table 2. All 74 isolates had distinct single band amplicons for the positive control loci *FEMI* (Jonkers and Rep 2009) and the *F. oxysporum*-specific portion of *EF α 1* (Haegi et al. 2013), confirming initial morphological

assignment of isolates to *F. oxysporum*. No isolates contained an amplicon for the *SIX4* primer pair (the sequenced strain FOL MN-25 (Ma et al. 2010) was isolated from these same fields and is race 3, and the lack of *SIX4* locus is expected for all nonpathogens and race 2 or 3 pathogens (Houterman et al. 2009; Lievens et al. 2009a).

SIX gene assays using published primers revealed that four apparent effector haplotypes were present among the 74 assayed soil isolates of *F. oxysporum* (Table 2). Effector haplotypes *SIX*₀ (no SIX genes, 35 isolates) and *SIX*₁₂₃₅₆₇ (all assayed SIX genes except *SIX4*, 36 isolates) were the most common, comprising 96% of the total assayed isolates (Table 2). Effector haplotypes *SIX*₂₃ (two isolates) and haplotype *SIX*₂₃₆ (one isolate) comprised the remaining 4% of the assayed isolates. In all, 36 of 74 isolates (49%) carried the full complement of SIX genes (except *SIX4*, see above), while 39 isolates (53%) had an amplicon present for at least two SIX genes. Both mating-type idiomorphs (*MAT1-1* and *MAT1-2*) were detected in the population, with 26 isolates (35%) containing an amplicon for *MAT1-1*, and 48 isolates (65%) containing an amplicon for *MAT1-2* (Table 2). No isolates containing both mating-type idiomorphs (*MAT1-1* + *MAT1-2*) were detected.

The association of both mating type and haplotype with sample location, and the association of mating type and haplotype were examined through the use of χ^2 tests (w/Yates's continuity correction) on contingency tables (Table 3), where the null hypothesis was no association between the two categorical variables. Due to their low frequency (expected frequencies <5), effector haplotypes *SIX*₂₃ and *SIX*₂₃₆ were excluded from this analysis. Collection location was associated with both haplotype ($\chi^2 = 11.46$, $P = 0.009$) and mating type ($\chi^2 = 11.70$, $P = 0.008$) (Fig. 1). Collection location 3 (in a row at the base of tomato plants in field 2) had a significantly higher proportion of effector haplotype *SIX*₁₂₃₅₆₇ than effector haplotype *SIX*₀ (Fig. 1). Of the 12 isolates assayed from location 3, 10 were effector haplotype *SIX*₁₂₃₅₆₇ (83% more than expected) and only 1 isolate had the *MAT1-1* mating-type idiomorph (76% less than expected) (Fig. 1).

All isolates had one of the two mating-type idiomorphs but these mating-type idiomorphs were unequally distributed among the effector haplotype groups. Of the 36 isolates in effector haplotype

TABLE 2. Isolates used in this study and results of SIX and MAT gene assays

Haplotype ^a	N	Isolate IDs ^b	Origin	Source	SIX1	SIX2	SIX3	SIX4	SIX5	SIX6	SIX7	MAT1	MAT2
<i>SIX</i> ₀ (MAT1) ^c	22	FOS 1-1, 1-11, 1-14, 1-26, 2-3, 2-4, 2-5, 2-6, 2-8, 2-15, 2-18, 2-20, 2-21, 3-1 , 4-6, 4-13, 4-18, 4-19 , 4-26, 4-27, 4-28, 4-29	Manatee County, FL	Soil	— ^d	—	—	—	—	—	—	+ ^e	—
<i>SIX</i> ₀ (MAT2)	13	FOS 1-2, 1-6, 1-7, 1-10, 1-13, 1-17, 1-21, 1-23 , 2-13, 2-16, 2-19, 2-22, 4-23	Manatee County, FL	Soil	—	—	—	—	—	—	—	—	+
<i>SIX</i> ₂₃ (MAT2)	2	FOS 3-15 , 4-2	Manatee County, FL	Soil	—	+	+	—	—	—	—	—	+
<i>SIX</i> ₂₃₆ (MAT1)	1	FOS 4-9	Manatee County, FL	Soil	—	+	+	—	—	+	—	+	—
<i>SIX</i> ₁₂₃₅₆₇ (MAT1)	3	FOS 2-9 ^f , 2-11 ^f , 2-17^f	Manatee County, FL	Soil	+	+	+	—	+	+	+	+	—
<i>SIX</i> ₁₂₃₅₆₇ (MAT2)	33	FOS 1-12, 1-18, 1-20, 1-22, 1-24, 1-27, 1-29, 2-1, 3-2, 3-3, 3-4, 3-5^f , 3-7, 3-8, 3-9, 3-10, 3-11, 3-12, 4-1, 4-4^f , 4-5, 4-7, 4-10, 4-11, 4-12, 4-14, 4-15, 4-16, 4-17, 4-20, 4-22, 4-24, 4-25	Manatee County, FL	Soil	+	+	+	—	+	+	+	—	+
<i>SIX</i> ₀ (MAT2)	1	FO-47 (biocontrol)	France	Plant	—	—	—	—	—	—	—	—	+
<i>SIX</i> ₀ (MAT1)	1	CL-57 (f. sp. <i>radicis-lycopersici</i>)	Collier County, FL	Plant	—	—	—	—	—	—	—	+	—
<i>SIX</i> ₁₂₃₅₆₇ (MAT2)	1	FOL MN-25^f (f. sp. <i>lycopersici</i>) race 3	Manatee County, FL	Plant	+	+	+	—	+	+	+	—	+
<i>SIX</i> ₁₂₃₅₆₇ (MAT1)	1	FOL 4287^f (f. sp. <i>lycopersici</i>) race 2	Spain	Plant	+	+	+	—	+	+	+	+	—

^a Haplotypes were labeled according to their apparent SIX gene complement, as indicated by PCR assays. Isolates with no detected amplicons were labeled *SIX*₀, while isolates with detected amplicons were labeled according to the SIX gene amplicon suite (for example, an isolate that was positive for amplicons for *SIX2* and *SIX3* would be labeled *SIX*₂₃).

^b Bold isolates were selected for pathogenicity tests against the susceptible tomato cultivar MoneyMaker. FOS isolates are *F. oxysporum* soil (FOS) isolates unique to this study.

^c Mating-type idiomorphs (MAT 1-1 and MAT 1-2) are abbreviated as MAT1 and MAT2, respectively, in this table.

^d — indicates no amplicon was detected.

^e + indicates amplicon was detected.

^f SIX genes were sequenced.

*SIX*₁₂₃₅₆₇, 32 (89%) had the *MAT*1-2 idiomorph, while the isolates in effector haplotype *SIX*₀ were more evenly distributed with regard to *MAT* idiomorph but the majority (22 of 35) had the *MAT*1-1 idiomorph (63%) (Fig. 1). Within *MAT* idiomorph groups, *MAT*1-1 strains had a higher than expected number of isolates belonging to effector haplotype *SIX*₀ (22 of 25 isolates, 88%), 81% higher than expected, while *MAT*1-2 strains had a higher than expected number of isolates belonging to effector haplotype *SIX*₁₂₃₅₆₇ ($\chi^2 = 20.80$, $P < 0.0001$) (Fig. 1).

Outgroup and control samples had amplicons consistent with those expected from sequence information and previous studies (Ma et al. 2010). FOL MN-25, isolated from the same field as these soil isolates in Manatee County, FL was an effector haplotype

*SIX*₁₂₃₅₆₇ strain with the *MAT*1-2 idiomorph. Reference *F. oxysporum* f. sp. *lycopersici* strain FOL 4287 also was an effector haplotype *SIX*₁₂₃₅₆₇ strain with the *MAT*1-1 idiomorph. FO47 was an effector haplotype *SIX*₀ strain with an *MAT*1-2 idiomorph, while CL-57 was an effector haplotype *SIX*₀ strain with the *MAT*1-1 idiomorph.

Pathogenicity tests. Pathogenicity tests were conducted on representative isolates from effector haplotypes *SIX*₀ (FOS 1-23, FOS 3-1, and FOS 4-19), *SIX*₂₃ (FOS 3-15), *SIX*₂₃₆ (FOS 4-9), and *SIX*₁₂₃₅₆₇ (FOS 2-17, FOS 4-4, and FOS 3-5), along with the sequenced pathogenic strains FOL 4287 and FOL MN-25 (both effector haplotype *SIX*₁₂₃₅₆₇) and the bioprotective strain FO47 (effector haplotype *SIX*₀). Plant weight was negatively correlated with disease index (DI) ($R^2 = 0.27$, $P < 2e-16$, Fig. 2A). Of the 717

TABLE 3. Isolate location, effector haplotype, and mating-type frequencies

Field	Haplotype location ^a	<i>SIX</i> ₀		<i>SIX</i> ₂₃		<i>SIX</i> ₂₃₆		<i>SIX</i> ₁₂₃₅₆₇		Total
		<i>MAT</i> 1 ^b	<i>MAT</i> 2	<i>MAT</i> 1	<i>MAT</i> 2	<i>MAT</i> 1	<i>MAT</i> 2	<i>MAT</i> 1	<i>MAT</i> 2	
Field 1	Interrow 1 (1-IR)	4	8	0	0	0	0	0	7	19
	Interrow 2 (2-IR)	9	4	0	0	0	0	3	1	17
Field 2	Row (3-R)	1	0	0	1	0	0	0	10	12
	Interrow (4-IR)	8	1	0	1	1	0	0	15	26
Total		22	13	0	2	1	0	3	33	74

^a Haplotypes were labeled according to their apparent *SIX* gene complement, as indicated by PCR assays. Isolates with no detected amplicons were labeled *SIX*₀, while isolates with detected amplicons were labeled according to the *SIX* gene amplicon suite (for example, an isolate that was positive for amplicons for *SIX*2 and *SIX*3 would be labeled *SIX*₂₃).

^b Mating-type idiomorphs (*MAT* 1-1 and *MAT* 1-2) are abbreviated as *MAT*1 and *MAT*2, respectively, in this table.

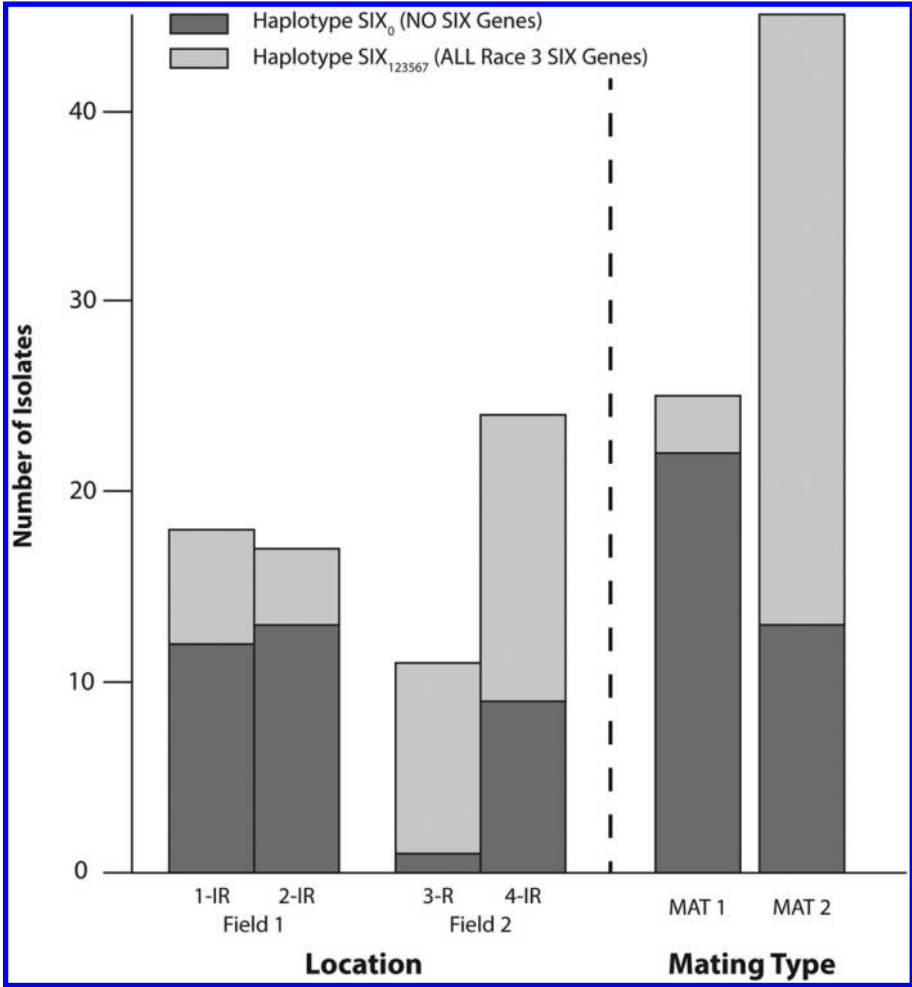


Fig. 1. Distribution of effector haplotypes *SIX*₀ and *SIX*₁₂₃₅₆₇ between collection locations (left) and mating-type idiomorphs (right). Abbreviations for collection locations are as follows: Field 1, interrow 1 = 1-IR; field 1, interrow 2 = 2-IR; field 2 row = 3-R; and field 2 interrow = 4-IR.

individual plants scored in four independent trials, 559 (78%), 37 (5%), 97 (14%), and 24 (3%) had disease indices of 0, 1, 2, and 3, respectively (Fig. 2). The 559 plants with disease indices of 0 had a mean weight of 5.57 ± 2.45 g; 37 plants with DI of 1 had a mean weight of 3.17 ± 2.14 g; 97 plants with DI of 2 had a mean weight of 2.37 ± 2.06 g; and 24 plants with a DI of 3 had a mean weight of 0.40 ± 0.36 g.

Only strains FOL 4287, FOL MN-25, and FOS 3-5 caused inoculated plants to have disease indices greater than zero (Fig. 2B). In four independent trials, FOL 4287 plants with disease indices of 0, 1, 2, and 3 made up 8, 27, 58, and 7%, respectively, of the total (Fig. 2B). FOL MN-25 had 0, 27, 57, and 17% of the total plants with disease indices of 0, 1, 2, and 3, respectively (Fig. 2B). Of the nine investigated soil isolates of *F. oxysporum*, only one (FOS 3-5, effector haplotype *SIX*₁₂₃₅₆₇) caused disease symptoms similar to those of FOL 4287 and FOL MN-25 (Fig. 2B and C). Soil isolate FOS 3-5 had 4, 17, 59, and 20% of inoculated plants with disease indices 0, 1, 2, and 3, respectively. All eight other tested soil isolates caused no disease symptoms in fully susceptible Moneymaker tomato plants (Fig. 2B).

Electrophoretic karyotyping (CHEF) and hybridization. Based on the results of gene assays and pathogenicity tests, three soil isolates were selected for electrophoretic karyotyping along

with the reference sequenced strains FOL 4287, FOL MN-25, and FO47. These soil isolates represented an effector haplotype *SIX*₁₂₃₅₆₇ pathogenic isolate (FOS 3-5), an effector haplotype *SIX*₁₂₃₅₆₇ isolate showing incongruence between *SIX* gene pattern and pathogenicity (FOS 4-4), and an effector haplotype *SIX*₀ nonpathogen (FOS 1-23).

Electrophoretic karyotyping revealed differing patterns of supernumerary chromosomes among all three soil isolates and previously sequenced strains (Fig. 3A). FOL 4287 has 15 chromosomes and contains all known *SIX* genes on the supernumerary chromosome 14 (Ma et al. 2010). Optical mapping of FOL MN-25 genomic scaffolds has demonstrated that this chromosome is largely homologous with FOL MN-25 chromosome 12 (Ma 2014; Ma et al. 2010). Based on our karyotyping results, a presumptive chromosome 12 homolog is also present in FOS 3-5, but notably absent in FOS 4-4 and FOS 1-23, both nonpathogenic strains (Fig. 3B). The smallest chromosome in FOL MN-25, chromosome 13 (0.89 Mb), is not present in FOS 3-5 or FOS 4-4, but FOS 1-23 does appear to have a chromosome of similar size. Chromosome 13 in FOS 3-5 is ~1 Mb in size and not shared with any of the other CHEF investigated soil isolates or FOL MN-25. The second smallest chromosome in FOS 1-23 is larger than most of the other

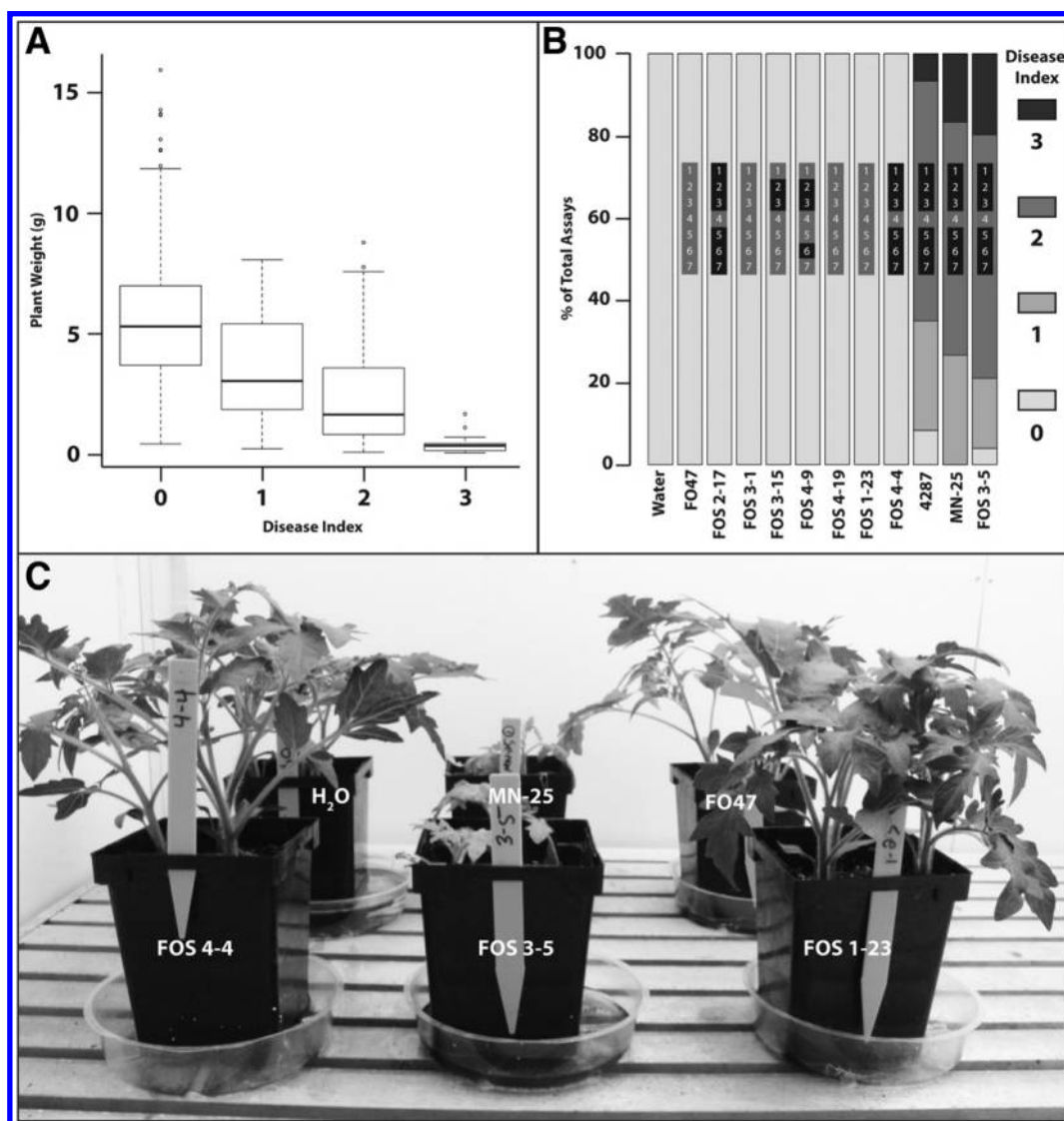


Fig. 2. **A**, Relationship of plant weight to disease index for all assayed plants in four independent inoculation trials. **B**, Distribution of disease index for assayed isolates and corresponding effector haplotype from *SIX* gene assays. Gray indicates absence of amplicon for *SIX* genes with corresponding number in the box, while black indicates presence of amplicon for *SIX* genes with corresponding number in the box. **C**, Representative phenology of plants inoculated with isolates causing visible disease (FOS 3-5 and MN-25, center), isolates not causing visible disease (FOS 4-4, front left; and FOS 1-23 and FO47, right) and water control (H₂O, back left). Note that not all isolates are represented in the image; however, all relevant data are presented in **B**.

small chromosomes in these isolates, ~1.5 Mb. Notably, the pattern of small supernumerary chromosomes is different for all tested isolates. The smallest chromosome in FOS 3-5 appears larger than the smallest chromosome in FOL MN-25 (chromosome 13) and appears absent in FOS 4-4. FOS 1-23, a non-pathogenic effector haplotype *SIX*₀ strain, also has two small chromosomes.

DNA amplicons specific for effector genes *SIX3* and *SIX6* were used as probes to determine the location of *SIX* genes on the chromosomes of pathogenic and nonpathogenic strains. These genes were selected because they are distantly located on chromosome 14 in the FOL 4287 genome (Table 4). All assayed *SIX* genes in FOL 4287 are located on chromosome 14, which has been identified as homologous to FOL MN-25 chromosome 12 (Ma 2014; Ma et al. 2010). Both the *SIX3* and *SIX6* probes localized to chromosomal bands of the size predicted for chromosomes 14 and 12, respectively, in FOL 4287 and FOL MN-25 (Fig. 3C and D). Additionally, these sequences were localized to a chromosome in FOS 3-5 corresponding in size to chromosome 12 in FOL MN-25. In contrast, both *SIX3* and *SIX6* gene sequences localized to a larger chromosome (2.2 to 3.5 Mb) in FOS 4-4 (Fig. 3E). Consistent with the *SIX* gene PCR assays, no sequence similarity was detected by Southern hybridization to

chromosomes for FOS 1-23 or FO47, the nonpathogenic *SIX*₀ isolates (Fig. 3E).

SIX gene sequence similarities among strains. The *SIX* genes from the four pathogenic strains analyzed for electrophoretic karyotype (FOL 4287 [*MAT1-1*, race 2], MN-25 [*MAT1-2*, race 3], FOS 4-4 [*MAT1-2*], FOS 3-5 [*MAT1-2*]), along with *SIX* genes from three *MAT1-1* isolates (FOS 2-9, FOS 2-11, and FOS 2-17) were amplified and sequenced. High quality sequence coverage of 83 to 91% of each gene was obtained. No SNPs were identified (no variation was detected) in any of gene sequences for all of the *SIX* genes except *SIX3* (Table 5). An SNP at position 121 was detected in *SIX3*. All sequenced *MAT1-2* isolates (FOL MN-25, FOS 4-4, and FOS 3-5) and a single *MAT1-1* isolate (FOS 4-9, haplotype *SIX*₂₃₆), had an adenine residue at position 121 (Table 5), corresponding to a known SNP (breaking the tomato resistance gene I-2) found in VCG0033 race 3 *F. oxysporum* f. sp. *lycopersici* isolates, such as FOL MN-25, which was isolated from these same production fields (Houterman et al. 2009). Except for FOS 4-9, other sequenced *MAT1-1* isolates (FOL 4287, FOS 2-9, FOS 2-11, and FOS 2-17, all haplotype *SIX*₁₂₃₅₆₇) had a guanine residue at position 121 (Table 5), suggesting this *SIX3* allele may have been derived from a FOL race 2 strain.

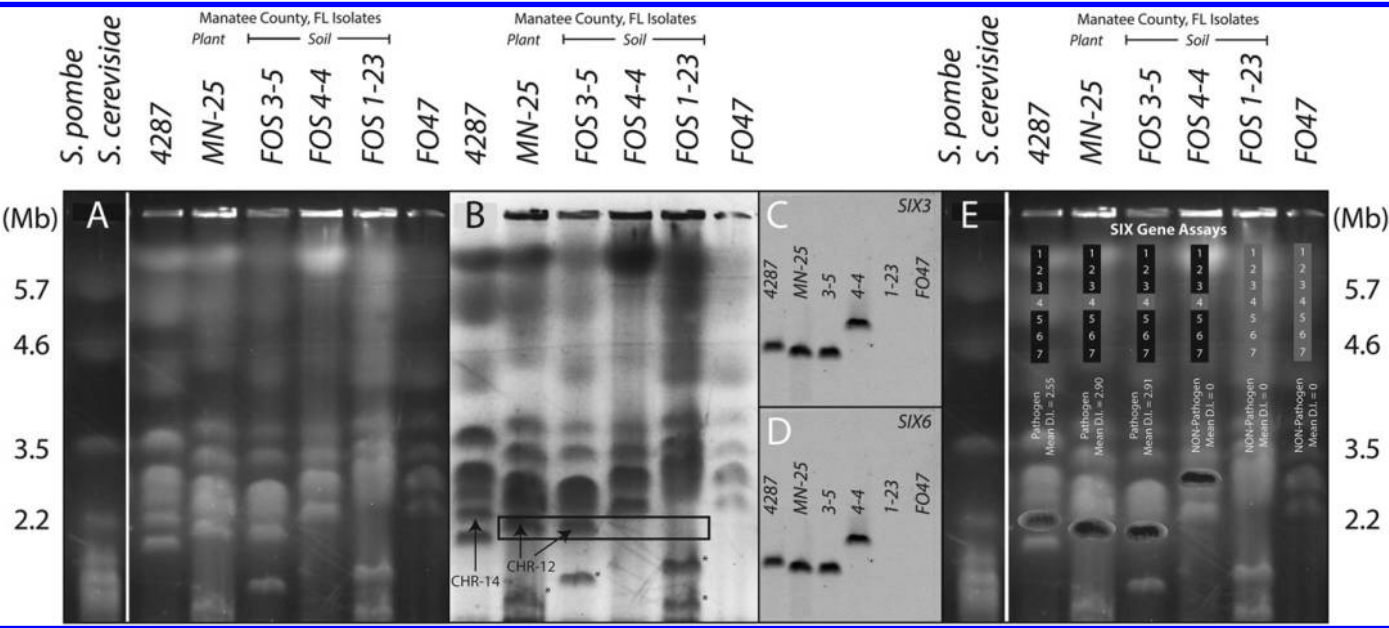


Fig. 3. A, Results of electrophoretic karyotyping by contour-clamped homogeneous electric field (CHEF) electrophoresis. B, Color-inverted image showing location of lineage specific chromosomes containing *SIX* genes (arrows) and small presumptive supernumerary chromosomes (asterisk). Known chromosome numbers containing *SIX3* and *SIX6* in two sequenced isolates (chromosome 14 in 4287 [CHR-14] and chromosome 12 in MN-25 [CHR-12]) are indicated, with chromosome 12 in FOS 3-5 predicted by size and synteny. The box indicates the absence of a similar-sized chromosome in FOS 4-4 and FOS 1-23. C, Southern hybridization with *SIX3* probe. D, Southern hybridization with *SIX6* probe. E, Scaled location of *SIX3* and *SIX6* probes on original CHEF image for comparison and corresponding effector haplotype from *SIX* gene assays. Gray bars indicate absence of amplicon for *SIX* genes, and black bars indicate presence of amplicon for *SIX* genes. Mean disease index (D.I.) for all tomato plants inoculated with each strain is presented.

TABLE 4. *SIX* genes and locations in genomes of FOL 4287 and FOL MN-25

Gene	FOL 4287				FOL MN-25			
	Chromosome ^a	Super contig	Position	Strand	Chromosome ^b	Super contig	Position	Strand
<i>SIX1</i>	14	36	116409–117263	POS	12	109	1278–2132	NEG
<i>SIX1H^c</i>	14	36	94690–95062	POS	12	96	26500–26872	NEG
			94512–94648				26914–27050	
<i>SIX2</i>	14	36	108933–109631	NEG	12	109	8766–9464	NEG
<i>SIX3</i>	14	36	5017–5484	POS	12	203	1551–2018	NEG
<i>SIX5</i>	14	36	3270–3795	NEG	12	203	3240–3765	NEG
<i>SIX6</i>	14	22	761478–762125	NEG	12	254	468–1115	NEG
<i>SIX7</i>	14	51	65216–65875	POS	12	96	10512–11171	POS

^a Chromosome determined from genomic analysis of FOL 4287 (Ma et al. 2010).

^b Chromosome predicted from comparative optical mapping of FOL 4287 and FOL MN-25 genomes (Ma 2014; Ma et al. 2010).

^c Pseudo *SIX1* gene (Rep et al. 2005).

Nitrate nonutilizing mutants and vegetative compatibility tests. Nitrate nonutilizing mutants were generated for FOL MN-25 and the three soil strains which were analyzed by electrophoretic karyotyping (FOS 1-23, FOS 3-5, and FOS 4-4). nit1 mutants were generated for all strains, while (due to their relative rarity) NitM mutants were generated only for FOS 3-5 and FOS 1-23 and nit3 mutants for FOS 3-5 and FOS 4-4. The scoring and results of these pairings are shown in Figure 4 and Table 6. These tests clearly place FOS 3-5 and FOS 4-4 in VCG 0033 (the known VCG for FOL MN-25), whereas FOS 1-23 generated wild-type growth only when paired with itself and thus is placed in a separate (unknown) VCG.

DISCUSSION

Our results reveal the dynamic nature of effector genes and widespread chromosomal polymorphisms among pathogenic and

nonpathogenic strains of *F. oxysporum* associated with soil in tomato fields undergoing a tomato wilt epidemic. A previous study (Lievens et al. 2009a) examined the presence of SIX genes among strains isolated from plants, either tomato wilt strains of *F. oxysporum*, or *F. oxysporum* strains with different host specificities from around the world. Only two SIX gene haplotypes were found: all tomato wilt strains contained all SIX genes (equivalent to effector haplotype SIX₁₂₃₅₆₇ in our study) and all other *F. oxysporum* strains, including the tomato crown rot strain *F. oxysporum* f. sp. *radicis-lycopersici*, contained no SIX genes (equivalent to effector haplotype SIX₀ in our study). Based on Lievens et al. (2009a), our expectation was to find either *F. oxysporum* strains fully pathogenic on tomato and having all SIX genes present or strains non-pathogenic to tomato and lacking all SIX genes.

Our findings were at odds with these expectations for several reasons. First, although primers were designed to amplify SIX genes specifically found in a *F. oxysporum* f. sp. *lycopersici* strain

TABLE 5. SIX gene amplicon sequence length and SNP observations

Primer pair	Predicted amplicon length (bp)	Consensus sequence length (w/Phred quality >20) ^a	SNPs detected	Predicted product sequenced
SIX1	992	898	–	91%
SIX2	749	623	–	83%
SIX3	608	515	A ₁₂₁ in FOS 4-9, FOS 4-4, FOS 3-5, MN-25; G ₁₂₁ in FOL 4287, FOS 2-9, FOS 2-11, FOS 2-17	85%
SIX4 ^b	967	–	NA	NA
SIX5	667	562	–	84%
SIX6	793	698	–	88%
SIX7	862	766	–	89%

^a Length of contiguous sequence obtained for which all analyzed isolates had a Phred quality score >20 (99% accuracy).

^b SIX4 amplicons were not obtained for any isolates.

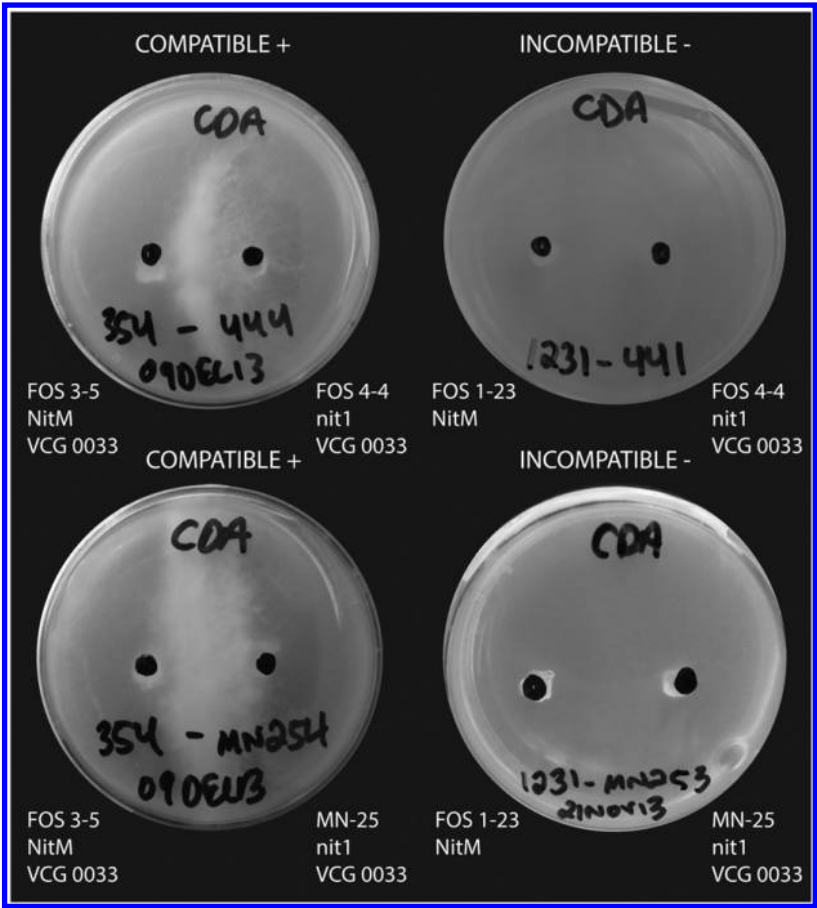


Fig. 4. Selected vegetative compatibility tests demonstrating scoring of compatible versus noncompatible mutants and assignment of vegetative compatibility groups.

isolated from this field (FOL MN-25), we observed a low frequency of strains having only two or three of the seven assayed SIX genes. Perhaps not surprising, tested strains having less than the full complement of seven SIX genes were nonpathogenic on tomato.

More surprising however, was that two of the three tested soil isolates having all race 3 SIX genes (FOS 2-17 and FOS 4-4) were also nonpathogenic to tomato. Only one of the assayed soil isolates with the full suite of assayed SIX genes (FOS 3-5) caused disease symptoms on susceptible tomato plants similar to known strains of *F. oxysporum* f. sp. *lycopersici*. Moreover, sequenced portions of SIX gene amplicons compared among the sequenced pathogens (FOL 4287 and FOL MN-25), the soil pathogen (FOS 3-5) and a soil nonpathogen (FOS 4-4) revealed no SNPs that might indicate loss or alteration of function in the FOS 4-4 SIX genes. Further, vegetative compatibility analysis revealed that FOS 4-4 belongs to the same VCG (0033) as FOL MN-25 and FOS 3-5 and thus is closely related to these pathogenic strains.

Another unexpected finding has to do with the distribution of mating-type idiomorphs among the soil isolates, especially those strains containing all SIX genes. A previous global survey of mating type in *F. oxysporum* isolates revealed a relatively even distribution of idiomorphs but slightly more isolates with the *MAT1-1* idiomorph (63% of 52 isolates, Lievens et al. 2009b). In contrast, the *MAT1-2* idiomorph is dominant in our isolate collection (48 isolates or 65%) and we provide evidence that MAT idiomorphs are highly correlated with effector haplotypes at the field scale. Nevertheless, given that *F. oxysporum* has no known sexual stage, we would have predicted that strains containing all SIX genes would likely be clonally derived from the pathogenic effector haplotype *SIX*₁₂₃₅₆₇ typified by FOL MN-25 (*MAT1-2*), isolated from diseased plants. Interestingly, 3 of the 35 strains having effector haplotype *SIX*₁₂₃₅₆₇ had the *MAT1-1* idiomorph. However, these three strains (FOS 2-9, FOS 2-11, and FOS 2-17) also had *SIX3* (AVR2) alleles consistent with the allele from FOL race 2 strains like FOL 4287 (Houterman et al. 2009) which also has the *MAT1-1* idiomorph. It is possible then that these nonpathogenic strains were actually derived from a FOL race 2 genotype, and that they persisted in the soil after I-2 resistant tomato varieties were deployed in this field.

Several hypotheses may account for the lack of pathogenicity in an isolate like FOS 4-4. The examination of the electrophoretic karyotypes of soil isolates showed size polymorphism for the chromosome encoding known effector genes. This chromosome (number 12 in the *F. oxysporum* f. sp. *lycopersici* pathogen FOL MN-25 and FOS 3-5) is roughly 2 Mb in size and corresponds to the 2.2 Mb chromosome 14 in FOL 4287 (Fig. 3). However, the nonpathogenic soil isolate FOS 4-4 lacks a chromosome in this size range. Southern hybridization using two spatially separated SIX genes (*SIX3* and *SIX6*) as probes showed that in FOS 4-4 these SIX genes are localized to a larger chromosome between 2.2 and 3.5 Mb in size (Fig. 3). This suggests several possibilities: (i) the translocation of the chromosome 12 homolog in isolate FOS 4-4 altered the genomic landscape and regulation of SIX genes that may

account for the isolate's loss of pathogenicity, (ii) that other (un-assayed in this study) important effectors (such as ORX1 or additional SIX genes (Laurence et al. 2015)) or regulatory elements (Nino-Sanchez et al. 2016) are missing in this strain, or (iii) that the chromosome 12 homolog in isolate FOS 4-4 is the result of a partial or "unsuccessful" transfer of pathogenicity-related genetic material.

Several important questions remain unresolved, such as the genetic (or epi-genetic) basis for nonpathogenicity of FOS 4-4, which perhaps will be best resolved through further comparative genomic studies between this isolate and FOL MN-25. Are there differences in SIX gene expression among isolates that could explain the different pathogenicity phenotypes? It is also currently unknown whether FOS 4-4 is able to infect the root, or whether it is deficient in other factors (perhaps transcription factors) related to host colonization. Exploring further effector haplotype *SIX*₁₂₃₅₆₇ yet nonpathogenic soil isolates such as FOS 2-17 may suggest whether there are single or multiple causes for the nonpathogenic phenotype.

Taken together, these results indicate that the presence of the SIX gene effectors alone is not sufficient to explain pathogenicity in *F. oxysporum* soil isolates. While previous studies have evaluated the presence and absence of these markers from strains isolated directly from plants, the free-living soil population of *F. oxysporum* offers an arena for the exchange of genetic material that leads to the genesis of pathogenic strains. Our results imply that genetic exchange in free-living soil populations of *F. oxysporum* may be highly dynamic, relatively frequent and should be considered in the development of models of disease risk. Particularly for *F. oxysporum*, which can survive as a free-living saprobe and exchange genetic material in the soil, looking solely at isolates from plant material may not capture this ongoing process.

ACKNOWLEDGMENTS

We thank L. R. Gale for conversations related to this study, H. Xayamongkhon and L. Lofgren for laboratory assistance, K. LaBine for assistance with isolate curation and sequencing, and R. Pettway for collecting and isolating *Fusarium* strains from soil samples. Sequencing and annotation of the reference strain FOL MN-25 was supported by Agriculture and Food Research Initiative Competitive Grant 2008-35600-04691 from the USDA National Institute of Food and Agriculture. Sequencing and annotation of the reference strain was funded by the USDA's National Institute for Food and Agriculture, grants 2011-35600-30379 and 2008-35604-18800. Other support was provided by grant 2011-67019-30200 from the USDA National Institute of Food and Agriculture and a National Science Foundation Graduate Research Fellowship to N. A. Jelinski.

LITERATURE CITED

- Alabouvette, C., Olivain, C., Migheli, Q., and Steinberg, C. 2009. Microbiological control of soilborne phytopathogenic fungi with special emphasis on wilt-inducing *Fusarium oxysporum*. New Phytol. 184:529-544.
- Arie, T., Kaneko, I., Yoshida, T., Noguchi, M., Nomura, Y., and Yamaguchi, I. 2000. Mating type genes from asexual phytopathogenic ascomycetes *Fusarium oxysporum* and *Alternaria alternata*. Mol. Plant-Microbe Interact. 13:1330-1339.
- Armstrong, G. M., and Armstrong, J. K. 1981. *Formae specialis* and races of *Fusarium oxysporum* causing wilt diseases. Pages 391-399 in: *Fusarium: Diseases, Biology and Taxonomy*. P. E. Nelson, T. A. Tousson, and R. J. Cook, eds. Pennsylvania University Press, University Park.
- Attitalla, I. H., Fatehi, J., Levenfors, J., and Brishammar, S. 2004. A rapid molecular method for differentiating two special forms (*lycopersici* and *radicis-lycopersici*) of *Fusarium oxysporum*. Mycol. Res. 108:787-794.
- Chakrabarti, A., Rep, M., Wang, B., Ashton, A., Dodds, P., and Ellis, J. 2010. Variation in potential effector genes distinguishing Australian and non-Australian isolates of the cotton wilt pathogen *Fusarium oxysporum* f. sp. *vasinfectum*. Plant Pathol. 60:232-43.
- Corrales-Escobosa, A. R., Wrobel, K., Landero Figueroa, J. A., Gutierrez Corona, J. F., and Wrobel, K. 2010. Effect of *Fusarium oxysporum* f. sp. *lycopersici* on the soil-to-root translocation of heavy metals in tomato plants susceptible and resistant to the fungus. J. Agric. Food Chem. 58:12392-12398.

TABLE 6. Vegetative compatibility pairing results

Isolate/mutant	FOS ^a 3-5/nitM ^b	FOS 1-23/nitM	FOS 3-5/nit3	FOS 4-4/nit3
FOS 4-4/nit1	+ ^c	- ^d	+	+
FOS 1-23/nit1	-	+	-	-
MN-25/nit1	+	-	+	+
FOS 3-5/nit1	+	-	+	+

^a FOS isolates are *F. oxysporum* soil (FOS) isolates unique to this study.

^b Nitrate nonutilizing mutant type, as determined by phenotyping growth on CDA, NO₂, and H_x media (Leslie 1990). NitM/nit1 mutations and nit1/nit3 mutations are complementary.

^c - indicates no observed vegetative compatibility, as determined by lack of dense zone of growth where hyphae from each isolate met on minimal medium.

^d + indicates vegetative compatibility, as determined by a zone of dense, aerial growth emerging from location where hyphae from each isolate met on minimal medium.

- De Cal, A., Pascual, S., Larena, I., and Melgarejo, P. 1995. Biological control of *Fusarium oxysporum* f. sp. *lycopersici*. Plant Pathol. 44:909-917.
- Elias, K. S., and Schneider, R. W. 1991. Vegetative compatibility groups in *Fusarium oxysporum* f. sp. *lycopersici*. Phytopathology 81:159-162.
- Fravel, D., Olivain, C., and Alabouvette, C. 2003. *Fusarium oxysporum* and its biocontrol. New Phytol. 157:493-502.
- Gale, L. R., Katan, T., and Kistler, H. C. 2003. The probable center of origin of *Fusarium oxysporum* f. sp. *lycopersici* VCG 0033. Plant Dis. 87:1433-1438.
- Garibaldi, A., Gilardi, G., and Gullino, M. L. 2004. Seed transmission of *Fusarium oxysporum* f. sp. *lactucae*. Phytoparasitica 32:61-65.
- Griekspoor, A., and Groothuis, T. 2005. 4Peaks. <http://nucleobytes.com/>
- Haegi, A., Catalano, V., Luongo, L., Vitale, S., Scotton, M., Ficcadenti, N., and Belisario, A. 2013. A newly developed real-time PCR assay for detection and quantification of *Fusarium oxysporum* and its use in compatible and incompatible interactions with grafted melon genotypes. Phytopathology 103:802-810.
- Houterman, P. M., Cornelissen, B. J. C., and Rep, M. 2009. Suppression of plant resistance gene-based immunity by a fungal effector. PLoS Pathog. 4: e1000061.
- Inami, K., Kashiwa, T., Kawabe, M., Onokubo-Okabe, A., Ishikawa, N., Rodriguez Perez, E., Hozumi, T., Aragon Caballero, L., Caceres de Baldarrago, F., Jimenez Roco, M., Madadi, K. A., Peever, T. L., Teraoka, T., Kodama, M., and Arie, T. 2014. The tomato wilt fungus *Fusarium oxysporum* f. sp. *lycopersici* shares common ancestors with nonpathogenic *F. oxysporum* isolates from wild tomatoes in the Peruvian Andes. Microbes Environ. 29:200-210.
- Irzykowska, L., and Kosiada, T. 2011. Molecular identification of mating type genes in asexually reproducing *Fusarium oxysporum* and *F. culmorum*. J. Plant Prot. Res. 51:405-409.
- Jonkers, W., and Rep, M. 2009. Mutation of CRE1 in *Fusarium oxysporum* reverts the pathogenicity defects of the FRP1 deletion mutant. Mol. Microbiol. 74:1100-1113.
- Jonkers, W., Xayamongkhon, H., Haas, M., Olivain, C., van der Does, H. C., Broz, K., Rep, M., Alabouvette, C., Steinberg, C., and Kistler, H. C. 2014. *EBR1* genomic expansion and its role in virulence of *Fusarium* species. Environ. Microbiol. 16:1982-2003.
- Kistler, H. C. 2001. Evolution of host specificity in *Fusarium oxysporum*. In: *Fusarium*: Paul E. Nelson Memorial Symposium, Pennsylvania State University-1997. B. A. Summerell, J. F. Leslie, D. Backhouse, W. L. Bryden, and L. W. Burgess, eds. American Phytopathological Society, St. Paul, MN.
- Klein, K. K., and Correll, J. C. 2001. Vegetative compatibility group diversity. In: *Fusarium*: Paul E. Nelson Memorial Symposium, Pennsylvania State University-1997. B. A. Summerell, J. F. Leslie, D. Backhouse, W. L. Bryden, and L. W. Burgess, eds. American Phytopathological Society, St. Paul, MN.
- Komada, H. 1975. A new selective medium for isolating *Fusarium* from natural soil. Rev. Plant Prot. Res. 8:114-125.
- Laurence, M. H., Summerell, B. A., and Liew, E. C. Y. 2015. *Fusarium oxysporum* f. sp. *canariensis*: Evidence for horizontal gene transfer of putative pathogenicity genes. Plant Pathol. 64:1068-1075.
- Leslie, J. F. 1990. Genetic exchange within sexual and asexual populations of the genus *Fusarium*. Pages 37-48 in: *Fusarium Wilt of Banana*. R. C. Ploetz, ed. American Phytopathological Society, St. Paul, MN.
- Leslie, J. F., and Summerell, B. A. 2006. The *Fusarium* Laboratory Manual. Blackwell Publishing, Ames, IA.
- Lievens, B., Houterman, P. M., and Rep, M. 2009a. Effector gene screening allows unambiguous identification of *Fusarium oxysporum* f. sp. *lycopersici* races and discrimination from other *formae speciales*. FEMS Microbiol. Lett. 300:201-215.
- Lievens, B., van Baarlen, P., Verreth, C., van Kerckhove, S., Rep, M., and Thomma, B. P. H. J. 2009b. Evolutionary relationships between *Fusarium oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radices-lycopersici* isolates inferred from mating type, elongation factor-1a and exopolygalacturonase sequences. Mycol. Res. 113:1181-1191.
- Long, A., Heitman, J., Tobias, C., Philips, R., and Song, B. 2013. Co-occurring anammox, denitrification, and co-denitrification in agricultural soils. Appl. Environ. Microbiol. 79:168-176.
- Ma, L.-J. 2014. Horizontal chromosome transfer and rational strategies to manage *Fusarium* vascular wilt diseases. Mol. Plant Pathol. 15:763-766.
- Ma, L.-J., Geiser, D. M., Proctor, R. H., Rooney, A. P., O'Donnell, K., Trail, F. 2013. *Fusarium* pathogenomics. Annu. Rev. Microbiol. 67:399-416.
- Ma, L.-J., van der Does, H. C., Borkovich, K. A., Coleman, J. J., Daboussi, M. J., and Di Pietro, A. 2010. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. Nature 464:367-373.
- Meldrum, R. A., Fraser-Smith, S., Tran-Nguyen, L. T. T., Daly, A. M., and Aitken, E. A. B. 2012. Presence of putative pathogenicity genes in isolates of *Fusarium oxysporum* f. sp. *cubense* from Australia. Australas. Plant Pathol. 41:551-557.
- Michielse, C. B., van Wijk, R., Reijnen, L., Manders, E. M., Boas, S., Olivain, C. 2009. The nuclear protein *Sge1* of *Fusarium oxysporum* is required for parasitic growth. PLoS Pathog. 5:e1000637.
- Nino-Sanchez, J., Casado-Del Castillo, V., Tello, V., De Vega-Bartol, J. J., Ramos, B., Sukno, S. A., and Maria Diaz Mínguez, J. 2016. The FTF gene family regulates virulence and expression of SIX effectors in *Fusarium oxysporum*. Mol. Plant Pathol. 17:1124-1139.
- O'Donnell, K., Kistler, H. C., Cigelnik, E., and Ploetz, R. C. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. USA 95:2044-2049.
- Rep, M., Van Der Does, H. C., and Cornelissen, B. J. 2005. Drifter, a novel, low copy hAT-like transposon in *Fusarium oxysporum* is activated during starvation. Fungal Genet. Biol. 42:546-553.
- Rocha, L. O., Laurence, M. H., Ludowici, V. A., Puno, V. I., Lim, C. C., Tesoriero, L. A., Summerell, B. A., and Liew, E. C. Y. 2016. Putative effector genes detected in *Fusarium oxysporum* from natural ecosystems of Australia. Plant Pathol. 65:914-929.
- Rosewich, U. L., Pettway, R. E., Katan, T., and Kistler, H. C. 1999. Population genetic analysis corroborates dispersal of *Fusarium oxysporum* f. sp. *radices-lycopersici* from Florida to Europe. Phytopathology 89:623-630.
- Schmidt, S. M., Houterman, P. M., Schreiver, I., Ma, L., Amyotte, S., Chellappan, B., Boeren, S., Takken, F. L. W., and Rep, M. 2013. MITEs in the promoters of effector genes allow prediction of novel virulence genes in *Fusarium oxysporum*. BMC Genomics 14:119.
- Shahi, S., Beerens, B., Bosch, M., Linmans, J., and Rep, M. 2016. Nuclear dynamics and genetic rearrangement in heterokaryotic colonies of *Fusarium oxysporum*. Fungal Genet. Biol. 91:20-31.
- Sievers, F., Wilm, A., Dineen, D. G., Gibson, T. G., Karplus, K., Li, W. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7:539.
- van Dam, P., Fokkens, L., Schmidt, S. M., Linmans, J. H., Kistler, H. C., Ma, L. J., and Rep, M. 2016. Effector profiles distinguish *formae speciales* of *Fusarium oxysporum*. Environ. Microbiol. 18:4087-4102.
- van der Does, H. C., Lievens, B., Claes, L., Houterman, P. M., Cornelissen, B. J. C., and Rep, M. 2008. The presence of a virulence locus discriminates *Fusarium oxysporum* isolates causing tomato wilt from other isolates. Environ. Microbiol. 10:1475-1485.