Specific PCR Detection of *Fusarium oxysporum* f. sp. *vasinfectum* California Race 4 Based on a Unique *Tfo1* Insertion Event in the *PHO* Gene

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Abstract

A highly virulent race 4 (Cal race 4) of *Fusarium oxysporum* f. sp. *vasinfectum* was identified in California cotton fields in 2001, and has since been found in increasing numbers of fields. Cal race 4 isolates contain a unique *Tfo1* transposon insertion in the *PHO* gene that was not found in other *F. oxysporum* f. sp. *vasinfectum* genotypes. Based on this insertion, a multiplex polymerase chain reaction method was developed to detect the Cal race 4 pathogen. A panel of *F. oxysporum* f. sp. *vasinfectum* isolates representing different vegetative compatibility groups (VCG) and DNA sequence types was assembled to test the specificity of the detection method. In all, 16 of 17 Cal race 4 isolates produced a 583-bp amplicon; the other isolate produced a 396-bp amplicon reflecting the

absence of the *Tfo1* insertion. This isolate was a moderately virulent pathogen among Cal race 4 isolates. In total, 80 other *F. oxysporum* isolates associated with cotton and 11 other formae speciales of *F. oxysporum* produced only the 396-bp amplicon. The method also distinguished Cal race 4 isolates from India race 4 isolates and China race 7 isolates, which did not possess the unique *Tfo1* insertion but otherwise had identical DNA sequences, and all belong to VCG0114. The method is capable of detecting the pathogen directly from infected stem tissues even before external symptom appears and, thus, provides an effective tool for timely identification of infested fields and seed lots, and should help reduce dissemination of Cal race 4 in the U.S. Cotton Belt.

Members of the *Fusarium oxysporum* species complex are common soil saprophytes, but are best known as vascular wilt and root rot pathogens in a broad range of plant species (Di Pietro et al. 2003; Gordon and Martyn 1997). Isolates have been subclassified into formae speciales based on their host range which generally is very limited (Snyder and Hansen 1940). Those isolates that attack cotton (*Gossypium* spp.) were designated as *F. oxysporum* f. sp. *vasinfectum* W. C. Snyder & H. N. Hansen. Isolates within *F. oxysporum* f. sp. *vasinfectum* have further been categorized into races based on their differential virulence to host genotypes (Armstrong and Armstrong 1960). Eight races have been designated thus far: races 1 and 2 from the United States, race 3 from Egypt, race 4 from India, race 5 from Sudan, race 6 from Brazil, and races 7 and 8 from China (Armstrong and Armstrong 1960, 1978; Armstrong and Armstrong 1958; Chen et al. 1985; Ibrahim 1966). Though the validity of race designations within *F. oxysporum* f. sp. *vasinfectum* is currently being challenged (Davis et al. 1996; Davis et al. 2006), these race designations are still in use today in literature, often based not on race concept but on DNA sequence identity or similarity with the reference race isolates. For example, “California race 3” isolates was much less virulent than the original India race 3 on *Gossypium barbadense* and their DNA sequences differed from that of Indian race 3 (Kim et al. 2005). Furthermore, race 5 was retracted when pathogenicity tests on cotton differential hosts and culture phenotypes indicated that it was identical to race 3 (Nirenberg et al. 1994). DNA sequence analysis also

confirmed that race 5 was indistinguishable from race 3 (Skovgaard et al. 2001).

Due to confusion and conflicts in the uniform application of the race concept within the currently available differential hosts, no more new races have been erected in recent decades (Davis et al. 1996; Holmes et al. 2009). Instead, new isolates have been simply designated as genotypes or biotypes based on DNA sequencing or biochemical characterization, respectively. Genetic variations among races, new biotypes, and genotypes have been assessed by DNA sequence and vegetative compatibility group (VCG) analyses (Davis et al. 1996; Fernandez et al. 1994; Kim et al. 2005; Skovgaard et al. 2001). Vegetative compatibility is governed by a number of *vic* loci in filamentous fungi and requires all the alleles at each locus to be the same in two compatible isolates (Leslie 1993). Thus, isolates in the same VCG may represent a clonal lineage in an asexual population such as *F. oxysporum*, or at least indicate close genetic relatedness (Puhalla 1985). Therefore, VCG analysis can provide a fine fingerprint of the isolates but lacks genetic relatedness information between two different VCG. Genomic information, obtained from limited stretches of sequences, may lack sufficient resolution to distinguish different VCG but reveals genetic relationships among VCG. Thus, combining these two analyses can provide very powerful tools to categorize different pathogenic *F. oxysporum* f. sp. *vasinfectum* isolates.

In 2001, highly virulent *F. oxysporum* f. sp. *vasinfectum* isolates were observed in Pima cotton (*G. barbadense*) on the west side of the San Joaquin Valley of California (Kim et al. 2005). These *F. oxysporum* f. sp. *vasinfectum* isolates occurred on heavy soils and did not require nematodes to cause severe disease. The newly found isolates were subsequently identified and designated as Cal race 4 based on sequence match to the race 4 reference isolate from India. The original race 4 from India was reported to infect cultivars of *G. arboreum* but not those of *G. barbadense* and *G. hirsutum* (Armstrong and Armstrong 1960). Race 4 in California also infected Upland cotton (*G. hirsutum*) varieties but with less severity (Kim et al. 2005). Race 7 from China had identical sequences compared with race 4 from India and California within three partial nuclear genes studied (Kim et al. 2005; Skovgaard et al. 2001) and caused disease in cultivars of *G. hirsutum*, *G. barbadense*, and *G. arboreum* (Chen et al. 1985). “Race 4-like” *F. oxysporum* f. sp. *vasinfectum* isolates were reported from Alabama and Mississippi

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Table 1. Fungal isolates used in this study

Isolate	Race; VCG ^b	Origin	Source	Multiplex PCR ^a	
				FovT-R	FovP-R
<i>F. oxysporum</i> from cotton					
Races 1, 2, 6 lineage ^c					
ATCC16421	1; 0111	South Carolina	G. M. Armstrong	—	+
AuSeed23	...; ...	Australia	Liu et al. 2011	—	+
CDR238	...; 01114	Arkansas	This study	—	+
ATCC36198	6; 0116	Brazil	G. M. Armstrong	—	+
CDR977	...; 01115	Alabama	This study	—	+
CDR982	...; 01115	Alabama	This study	—	+
CDR1034	...; 01115	Texas	This study	—	+
CDR960	...; 01116	Alabama	This study	—	+
CDR965	...; 01116	Alabama	This study	—	+
ATCC46644	...; 01117A	California	G. M. Armstrong	—	+
KL11-202	...; 01117A	Alabama	K. S. Lawrence	—	+
CA-10	...; 01117A	Kern Co., CA	Kim et al. 2005	—	+
CDR194	...; 01117A	Florida	This study	—	+
CDR43	...; 01117A	Louisiana	This study	—	+
CDR5	...; 01117A	Mississippi	This study	—	+
CDR219	...; 01117A	North Carolina	This study	—	+
CDR1023	...; 01117A	Texas	This study	—	+
CDR166	...; 01117B	Alabama	This study	—	+
CDR4	...; 01117B	California	This study	—	+
CDR84	...; 01117B	Georgia	This study	—	+
CDR235	...; 01117B	Louisiana	This study	—	+
CDR104	...; 01117B	South Carolina	This study	—	+
CDR218 (Fov218) ^d	...; 01117B	North Carolina	Liu et al. 2011	—	+
CDR927	...; 01117B	Texas	This study	—	+
ATCC24907	...; 01117C	California	S. Tao	—	+
KL11-1125	...; 01117C	Alabama	K. S. Lawrence	—	+
CDR252	...; 01117C	Arkansas	This study	—	+
CDR234	...; 01117C	Louisiana	This study	—	+
CDR930	...; 01117C	Texas	This study	—	+
ATCC16611	2; 0112	South Carolina	G. M. Armstrong	—	+
KL11-304	...; 0112	Alabama	K. S. Lawrence	—	+
KL11-402	...; 0112	Alabama	K. S. Lawrence	—	+
CDR1006	...; 0112	Arkansas	This study	—	+
CDR280	...; 0112	Mississippi	This study	—	+
CDR932	2; 0112	Texas	This study	—	+
CDR1131	...; ...	Louisiana	This study	—	+
CBS116613	...; 0115	Ivory Coast	Abo et al. 2005	—	+
CBS116623	...; 01110	Ivory Coast	Abo et al. 2005	—	+
Race 8 lineage					
NRRL31665	8; 01119	China	Skovgaard et al. 2001	—	+
CDR957	8; 01119	Alabama	This study	—	+
CDR958	8; 01119	Alabama	This study	—	+
CDR1073	8; 01119	Alabama	This study	—	+
KL10-101	...; 01119	Alabama	K. S. Lawrence	—	+
KL10-308	...; 01119	Alabama	K. S. Lawrence	—	+
CDR260	8; 01119	Arkansas	This study	—	+
CA-1A (CA-1) ^e	NP/SI	Tulare Co., CA	Kim et al. 2005	—	+
CA-1B (CA-1) ^e	8; 01119	Tulare Co., CA	Kim et al. 2005	—	+
CDR83	...; 01119	Georgia	This study	—	+
CDR233	...; 01119	Louisiana	This study	—	+
CDR203	...; 01119	South Carolina	This study	—	+
CDR943	...; 01120	Alabama	This study	—	+
CDR961	...; 01120	Alabama	This study	—	+

(continued on next page)

^a Multiplex PCR with primers FovP-F, FovP-R, and FovT-R; + denotes presence of the band and – denotes absence of the band; FovT-R column indicates the 583-bp amplicon from FovP-F and FovT-R while FovP-R column indicates the 396-bp amplicon from FovP-F and FovP-R.

^b VCG = vegetative compatibility group. NP = nonpathogenic in our pathogenicity assays on cotton. Isolates were obtained from diseased cotton tissues or soils in the rhizosphere of cotton. SI = heterokaryon self-incompatible.

^c Lineage: phylogenetic lineage based on concatenated sequence data of translation elongation factor (*EF-1 α*), phosphate: H⁺ Symporter (*PHO*), and β -tubulin (*BT*) genes.

^d Original name given in the parenthesis.

^e Two morphologically different isolates (A and B) were derived from the original culture in parentheses.

^f This California isolate with original designation of “91-1” was given a new name “11” and placed into race 3 group by Davis et al. (2003), with S. N. Smith as one of the authors. It was later designated as CA-11 by Kim et al. (2005). However, Fernandez et al. (1994) obtained isolate “91-1” from S. N. Smith, placed it into races 1, 2, 6 group (race A), and assigned VCG0119 to this isolate. This apparent conflict raises doubt as to the identity of the isolate “91-1” used by Fernandez and the validity of the assigned vegetative compatibility group (VCG) designation.

Table 1. (continued from preceding page)

Isolate	Race; VCG ^b	Origin	Source	Multiplex PCR ^a	
				FovT-R	FovP-R
KL11-205	...; 01120	Alabama	K. S. Lawrence	–	+
KL11-305	...; 01120	Alabama	K. S. Lawrence	–	+
KL11-408	...; 01120	Alabama	K. S. Lawrence	–	+
Races 4 and 7 lineage					
ATCC16613	4; 0114	India	G. M. Armstrong	–	+
BBA69519	4; 0114	India	Abo et al. 2005	–	+
BBA69520	4; 0114	India	Abo et al. 2005	–	+
BBA69521	4; 0114	India	Abo et al. 2005	–	+
NRRL31673	4; 0114	India	Skovgaard et al. 2001	–	+
NRRL31669	4; 0114	Unknown	Skovgaard et al. 2001	–	+
NRRL25433	7; 0114	China	Skovgaard et al. 2001	–	+
NRRL31672	7; 0114	China	Skovgaard et al. 2001	–	+
CA-9	4; 0114	Kings Co., CA	Davis et al. 2003; Kim et al. 2005	+	–
CA-12	4; 0114	Fresno Co., CA	Kim et al. 2005	+	–
CA-14A (CA-14) ^e	4; 0114	Fresno Co., CA	Kim et al. 2005	+	–
CA-14B (CA-14) ^e	...; SI	Fresno Co., CA	Kim et al. 2005	+	–
CA-S9	4; 0114	Fresno Co., CA	Kim et al. 2005	+	–
MD305	4; 0114	Kern Co., CA	R. M. Davis	+	–
MD306	4; 0114	Kern Co., CA	R. M. Davis	+	–
MD307	4; 0114	Kern Co., CA	R. M. Davis	+	–
MD308	4; 0114	Kern Co., CA	R. M. Davis	+	–
MD309	4; 0114	Merced Co., CA	R. M. Davis	+	–
MD310	4; 0114	Kern Co., CA	R. M. Davis	+	–
MD311	4; 0114	Kings Co., CA	R. M. Davis	+	–
MD312	4; 0114	Merced Co., CA	R. M. Davis	–	+
MD313	4; 0114	Kings Co., CA	R. M. Davis	+	–
MD314	4; 0114	Kern Co., CA	R. M. Davis	+	–
MD315	4; 0114	Kern Co., CA	R. M. Davis	+	–
MD316	4; 0114	Kings Co., CA	R. M. Davis	+	–
MD317	4; 0114	Kings Co., CA	R. M. Davis	+	–
AL-KL1	NP	Alabama	Bennett et al. 2013	–	+
AL-KL11	NP	Alabama	Bennett et al. 2013	–	+
MS-GL10	NP	Mississippi	Bennett et al. 2013	–	+
Race 3 lineage					
ATCC16612	3; 0113	Egypt	G. M. Armstrong	–	+
CA-11	...; (0119) ^f	Tulare Co., CA	Davis et al. 2003; Fernandez et al. 1994; Kim et al. 2005	–	+
CA-3	NP	Tulare Co., CA	Kim et al. 2005	–	+
CDR1072	...; 01118	Alabama	This study	–	+
CDR978	...; 01118	Alabama	This study	–	+
AuSeed5	...; 01113	Australia	Liu et al. 2011	–	+
CDR88	NP	Florida	This study	–	+
CDR538	NP	Texas	This study	–	+
AuSeed14	...; ...	Australia	Liu et al. 2011	–	+
Au lineage					
AuK24232	...; 01111	Australia	Liu et al. 2011	–	+
AuK24288	...; 01111	Australia	Liu et al. 2011	–	+
AuK24299	...; 01111	Australia	Liu et al. 2011	–	+
AuK24301	...; 01111	Australia	Liu et al. 2011	–	+
<i>F. oxysporum</i> f. sp. <i>conglutinans</i>					
PHW808	2; 0101	California	Kistler et al. 1991	–	+
<i>F. oxysporum</i> f. sp. <i>raphani</i>					
PHW815	...; 0102	France	Kistler et al. 1991	–	+
<i>F. oxysporum</i> f. sp. <i>pisi</i>					
HdV247	5; ...	Unknown	Kistler et al. 1991	–	+
<i>F. oxysporum</i> species complex 3a					
FOSC 3-a	...; ...	Massachusetts	Anonymous	–	+
<i>F. oxysporum</i>					
Fo47	...; ...	France	Alabouvette et al. 1987	–	+
<i>F. oxysporum</i> f. sp. <i>lycopersici</i>					
MN25	3; 0033	Florida	Anonymous	–	+
4287	2; 0030	Spain	Di Pietro and Roncero 1996	–	+
<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>					
CL57	...; 0094	Florida	Anonymous	–	+

(continued on next page)

Table 1. (continued from preceding page)

Isolate	Race; VCG ^b	Origin	Source	Multiplex PCR ^a	
				FovT-R	FovP-R
<i>F. oxysporum</i> f. sp. <i>melonis</i> NRRL26406	...; ...	Mexico	Ma et al. 2014	–	+
<i>F. oxysporum</i> f. sp. <i>cubense</i> II5	4; 01213	Indonesia	Anonymous	–	+
<i>F. verticillioides</i> 7600	...; ...	California	Anonymous	–	+
<i>F. graminearum</i> PH-1	...; ...	Michigan	Anonymous	–	–
<i>F. semitectum</i> CDRFsGA2	...; ...	Georgia	This study	–	–
<i>F. equiseti</i> CDRFe1	...; ...	Texas	This study	–	–
<i>F. solani</i> f. sp. <i>pisi</i> 77-13-4	...; ...	Northeastern United States	Coleman et al. 2009	–	–
<i>Verticillium dahlia</i> V-76	...; 1A	Mexico	Puhalla and Hummel 1983	–	–
<i>Thielaviopsis basicola</i> CDRTb1	...; ...	Texas	This study	–	–
<i>Chaetomium funicola</i> CDRCf1	...; ...	Texas	This study	–	–
<i>Phoma exigua</i> CDRPe1	...; ...	Texas	This study	–	–
<i>Aspergillus tubingensis</i> CDRAt01	...; ...	Louisiana	Crutcher et al. 2014	–	–
<i>Penicillium chrysogenum</i> CDRPe1	...; ...	Texas	F. K. Crutcher	–	–
<i>Tricoderma virens</i> Gv29-8	...; ...	Texas	Dave et al. 1994	–	–
<i>Talaromyces verruculosus</i> CDRTv1	...; ...	Texas	F. K. Crutcher	–	–

but these isolates were weak pathogens and were distinguished from race 4 by DNA sequences (Bennett et al. 2013) or vegetative compatibility (this study).

Since 2001, Cal race 4 isolates have been detected in increasing numbers of fields in California but, currently, are confined to that state. With inoculum level buildup, even tolerant varieties can sustain severe yield loss. Lack of resistant cultivars and persistence of the fungus in the field for decades, even in the absence of the cotton host, has increased concern about this race spreading throughout the U.S. Cotton Belt. Moreover, the presence of many possible mechanisms for fungal spore dispersal, including seedborne dissemination and soil and plant debris movement, make containment of the organism problematic (Davis et al. 2006; Liu et al. 2011; Smith and Snyder 1975). Therefore, a reliable detection method is imperative for early identification of infested fields and seed lots in order to prevent further movement of Cal race 4.

A primer pair designed to be race 4-specific has been developed to detect the presence of race 4 in cotton by polymerase chain reaction (PCR) (Yang et al. 2006) and a commercial race 4 detection kit has been developed with modified primers for use in an isothermal recombinase polymerase amplification reaction based on the same primer pair (Doan et al. 2014). However, both the PCR and the commercial kit showed positive reactions on the India race 3 and China race 7 reference isolates (Crutcher et al. 2016; Yang 2006). In this report, we describe a unique *TfoI* transposon insertion in the *PHO* gene in the Cal race 4 isolates. This insertion event was not present in any other known races or genotypes of *F. oxysporum* f. sp. *vasinfectum* isolates, and was used for the development of a Cal race 4-specific detection method using a multiplex PCR platform. The method was validated with a panel of *F. oxysporum* f. sp. *vasinfectum* isolates representing all known races and sequence types. These isolates were selected from initial VCG analysis of a large collection of *F. oxysporum* f. sp. *vasinfectum* isolates

followed by DNA sequence analysis of several isolates within each VCG.

Materials and Methods

Fungal isolates. Fungal isolates used in this study are summarized in Table 1. Isolates were selected after initial screening with VCG analysis of *F. oxysporum* f. sp. *vasinfectum* isolates from our and others fungal collections comprising more than 2,000 isolates. Within each VCG, several isolates (preferably from different geographic locations) were sequenced to identify all the possible genotypes for inclusion in the test panel. Thus, isolates represent all the known genetic diversity (17 VCG and 3 unnamed single-member VCG) within *F. oxysporum* f. sp. *vasinfectum* and geographic locations where cotton is grown in the United States. All the known race reference isolates and biotypes collected from major cotton-growing areas around the world were included. Each of the four Cal race 4 isolates with “CA” designations were collected from different fields in Fresno and Kings Counties of California’s San Joaquin Valley prior to 2003 (Davis et al. 2003). Thirteen Cal race 4 isolates with “MD” designations were collected in 2014 from Kern, Kings, and Merced Counties in the San Joaquin Valley and were kindly provided by Dr. R. M. Davis (University of California, Davis). Most of California’s cotton is grown in Fresno, Kings, and Kern Counties. Additional isolates representing other forma speciales of the *F. oxysporum* species complex, other distantly related *Fusarium* spp., as well as cotton-associated and soil-inhabiting fungal species also were part of this study. Pathogenicity of the cotton *F. oxysporum* isolates was evaluated by us or by the isolate contributors. All isolates were found to be pathogenic, unless otherwise noted, via stem puncture or soil infestation assays with or without root-knot nematodes (data not shown).

Isolate stocks were grown on 0.2× potato dextrose agar (PDA; Difco Laboratories, Detroit) plates amended with tetracycline (50 mg/liter) and chloramphenicol (100 mg/liter). After a round of

single-spore isolation, cultures were grown on 0.2× PDA plates for 7 days at 24°C. Glycerol stocks (30% glycerol [vol/vol]) for each culture were made and stored at –80°C.

Infection assay. Cotton seed (*G. hirsutum* L. ‘Coker 312’) were germinated by chipping the seed near the chalaza, wrapping them in filter paper, and placing them in a cup containing 10 ml of water. The cup was placed in a 30°C incubator for 24 h, then transferred to a 16°C room for an additional 24 h. Seedlings with a radicle length of about 1.3 to 2.5 cm were planted in plastic cups containing sterile 3:1 sand-loam (vol/vol, 500 ml total) with a core of sand-loam (50 g) mixed with cotton root powder (1.5 g). Pots were then inoculated at five locations, each with 1 ml (1×10^6) of *F. oxysporum* f. sp. *vasinfectum* CA-9 fungal spores (or water as negative control) in a 2-cm radius from the planted seedling. Pots were placed and maintained in a growth chamber with a 13-h day (6,000 lux) at 23 and 18°C (day and night, respectively) for 3 or 6 weeks. Disease severity was then visually evaluated on a rating scale of 0 to 4, where 0 = no symptoms; 1 = growth slightly suppressed or mild yellowing compared with healthy control with no more than 2 leaves wilted; 2 = severe yellowing and growth suppressed with more than 2 leaves wilted but at least 25% of leaves unaffected; 3 = all leaves showed wilting but at least one leaf not completely wilted (epinasty only, with no chlorotic mottling) and the top terminal of plant still upright; and 4 = all leaves completely wilted, plant bent at the top or dead. Cotton stems were cut about 1.5 cm in length just above the soil level from diseased and healthy (mock-inoculated) plants, surface sterilized, rinsed with sterile water, and flash frozen in liquid nitrogen for DNA extraction. The experiments were repeated.

Vegetative complementation. Vegetative compatibility of the isolates was tested according to Puhalla (1985) and Correll et al. (1987). Single-spore isolates were grown on freshly prepared potato sucrose agar (Puhalla 1985) containing 2% sodium chlorate (PSAC). Fast-growing sectors or dense circular areas within colonies were subcultured to new PSAC. After 3 to 4 days, hyphal fronts from subcultures were transferred to nitrate medium. Nitrate-nonutilizing (*nit*) mutants were identified using the media of Puhalla and Spieth (1983). Complementation among *nit-1*, *nit-3*, and *nit-M* mutants was first tested within an isolate to confirm that it was heterokaryon self-compatible and then among confirmed isolates to determine relationships.

DNA extraction. Fungal tissue was grown in liquid yeast extract peptone dextrose medium and incubated at 24°C for 5 days without shaking. Tissue was harvested on a sterile filter paper (Whatman, Maidstone, England) by vacuuming out the liquid and immediately

flash freezing in liquid nitrogen. Fungal DNA was isolated by a modified miniprep procedure (Woloshuk et al. 1989) and resuspended in sterile distilled water.

For DNA extraction from both the infected and healthy plant tissues, a procedure of Li et al. (2001) was utilized, with the following modifications: the extraction buffer was not used; instead, 0.03% β-mercaptoethanol (vol/vol) was added to the lysis buffer and this was then added to the frozen ground plant tissue. Additionally, all centrifugation steps were reduced to 10 min. DNA was resuspended in distilled water.

DNA concentrations were determined via NanoDrop (Thermo Scientific, Wilmington, DE) and integrity was analyzed by gel electrophoresis. Working DNA dilutions (50 ng/μl) were made and used throughout this study unless specified.

Sequencing and Phylogenetic analysis. Portions of three nuclear genes—translation elongation factor (*EF-1α*), phosphate: H⁺ symporter (*PHO*), and β-tubulin (*BT*)—were amplified using primers listed in Table 2. *PHO* with a *Tfo1* insertion was amplified in four overlapping fragments with primer pairs PHO48F and Tfo1451R, FovP-F and Tfo1919R, Tfo1730F and FovP-R, and Tfo2773F and PHO2034R (Table 1; Supplementary Table S1). Amplification was carried out in 100-μl reaction mixtures containing 100 ng of template DNA, 1.5 mM MgCl₂, 200 μM dNTP, 200 nM primers, and 2.5 U of GoTaq DNA polymerase in 1× GoTaq Flexi Buffer (Promega Corp., Madison, WI) and performed in an Eppendorf Mastercycler (Eppendorf, Westbury, NY) with the following program: initial denaturing at 94°C for 2 min; 35 cycles of denaturing at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 2 min; and final extension at 72°C for 5 min. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The same primers used for amplification and additional internal primers listed in Supplementary Table S1 were used to sequence the purified amplicons using the BigDye Terminator kit (v3.1; Perkin-Elmer, Boston) in an Eppendorf Mastercycler with the following cycling parameters: initial denaturation at 96°C for 2 min; 40 cycles of denaturation at 96°C for 15 s and annealing at 55°C for 15 s; and extension at 60°C for 4 min. Sequencing reaction mixtures were purified using Micro Bio-Spin P-30 spin columns (Bio-Rad, Hercules, CA) and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Texas A&M University Gene Technology Laboratory. Sequences were edited and aligned using Sequencher (version 4.8; Gene Codes, Ann Arbor, MI), and deposited in GenBank with the corresponding accession numbers listed in Supplementary Table S2.

DNA sequences were aligned using the Clustal W program (Thompson et al. 1994) and adjusted manually. Phylogenetic analyses were performed using the neighbor-joining method (Saitou and Nei 1987) implemented in the phylogenetic analysis program of MEGA 6.0 (Tamura et al. 2007) based on concatenated sequences of *EF-1α*, *PHO*, and *BT* genes of the isolates investigated in this study and isolates representing known genotypes. The *Tfo1* insertion sequence was coded as a single character in the *PHO* sequence. Sequences were compared with those of *F. oxysporum* strains and *F. verticillioides* strain 7600 (NCBI genome accession numbers: GCA_000149555.1, GCA_000149955.2, GCA_000259975.2, GCA_000260075.2, GCA_000260155.3, GCA_000260195.2, GCA_000260215.2, GCA_000260235.2, GCA_000260495.2, GCA_000271705.2, GCA_000271745.2). Kimura’s two-parameter distance option and pairwise deletion of gap option were used. Statistical support for the derived tree was obtained by running 1,000 bootstrap replicates.

Genome Walking. Insertion sequence of the *Tfo1* within the *PHO* gene was captured using a Genome Walker kit (Clontech Laboratories, Mountain View, CA) following the manufacturer’s protocols. Primers PHO-wR and PHO-w2R (Table 2) were used with AP1 and AP2 primers, respectively, to amplify the five libraries of the kit. The amplified fragments were resolved, purified, and sequenced as described above.

Detection of *Tfo1* insertion in *PHO*, sequence analysis, and amplification. *PHO* nucleotide sequences were aligned using Sequencher 4.8 software. Primers were designed manually and in silico based on CA-9 strain sequences and other *PHO*-sequenced

Table 2. DNA primers used in this study

Name ^a	Sequence (5′-3′)	Reference
EF-1α		
EF-1A	CACCTTAACGTCGTCGTCATC	Liu et al. 2011
EF1885R	CGAGTGCTCATGCCAACAT	This study
<i>PHO</i>		
PHO48F	TGCCTTCCACAACCTTTCACA	This study
PHO2034R	TCTCAACCAATCAAGGAATGTT	This study
β-Tubulin (BT)		
BT-100F	CCGTCAAAGCTAGGTCTACTTCA	This study
BT1828R	TGGAATTTTATTCGGCTCAT	This study
<i>PHO</i> genome walk		
PHO-wR	TCGCCCTTGAGTGTACATGAAC TACTCG	This study
PHO-w2R	AAGAAAGGGGATAATCACCACC AATACC	This study
<i>PHO-Tfo1</i> detection		
FovP-F	GGCCGATATTGTCGGTCGTA	This study
FovP-R	CTCCAGTGCAGTGCTTGGA	This study
FovT-R	ATCTGTCTTTCGTCGGCAAT	This study
Cellulose		
synthase A1		
CelA1-F	GGATCTGCACCCATCAATCT	Xin et al. 2003
CelA1-R	GCAAAGAGATGGGCTGAAAC	Xin et al. 2003

^a EF-1α = elongation factor-1α and *PHO* = phosphate: H⁺ symporter.

fragments from diverse *F. oxysporum* f. sp. *vasinfectum* isolates using the Primer-BLAST web-based platform (Ye et al. 2012). For amplification, the same GoTaq PCR mix conditions described above were used, with the exception of the addition of 0.1 μ M each FovP-F, FovP-R, and FovT-R primer and 50 ng of DNA in a 25- μ l reaction, unless otherwise specified. Negative controls without DNA template were included in every experiment to test for contamination or nonspecific amplification. After PCR, amplification products were resolved in an ethidium bromide prestained 1% agarose-Tris-acetate-EDTA gel at 6 V/cm for 35 min and photographed. To check the suitability of the plant sample's total DNA, primers CelA1-F and CelA1-R were used to amplify a 401-bp product of cotton's cellulose synthase A1 gene.

Results

A *Tfo1* transposable element resides in the *PHO* gene of *F. oxysporum* f. sp. *vasinfectum* Cal race 4 isolates. Attempts to amplify the *PHO* gene of Cal race 4 isolates failed to yield an amplicon using Taq polymerase if the primer pair surrounded base position 462. Amplicons for both the upstream region and the downstream region around position 462 had sequences identical to the corresponding *PHO* sequences of the race 4 reference isolate, indicating the presence of either a large insertion or a translocation in the Cal race 4 isolates. Genome walking upstream from the downstream side of position 462 generated a sequence about 1 kb in size that was more than 99% identical to that of the *F. oxysporum* transposon *Tfo1* (Okuda et al. 1998). *Tfo1*-based primers along with *PHO*-based primers (Table 2) enabled sequencing of the complete *Tfo1* insert and the surrounding *PHO* gene sequences. The insertion was 2,763 bp long and was located 463 bp upstream from the *PHO* start codon. None of other *F. oxysporum* f. sp. *vasinfectum* isolates sequenced, including the reference isolates India race 4 and China race 7, had the insertion at this position (Fig. 1). Using the junction sequence of *PHO* and *Tfo1*, *PHO* sequence-specific primers upstream and downstream of the *Tfo1* insertion (FovP-F and FovP-R; Fig. 2A) as well as one within the *Tfo1* open reading frame (FovT-R) were designed and tested (Table 2). Primer pair FovP-F and FovP-R was based on the

conserved sequences of *F. oxysporum* isolates and produced a 396-bp amplicon. Primer combination FovP-F and FovT-R produced a 583-bp amplicon (Fig. 2A). The 396-bp amplicon is only produced when a *Tfo1* insertion is not present in *PHO*, whereas a *Tfo1* insertion in *PHO* produces a 583-bp band. Based on this data, a multiplex PCR assay where the three primers were included was designed. No cross-reactivity was observed among primer pairs and only one band was produced per reaction on all tested *F. oxysporum* isolates.

Assessment of genetic diversity within *F. oxysporum* f. sp. *vasinfectum* and selection of a panel of *F. oxysporum* f. sp. *vasinfectum* isolates to validate Cal race 4-specific detection method. Genetic diversity within *F. oxysporum* f. sp. *vasinfectum* was assessed by VCG and sequence analysis. In all, 97 *F. oxysporum* isolates from cotton were selected after initial screening with VCG analysis of more than 2,000 isolates from our and other fungal collections, followed by sequencing several isolates within each VCG to represent all the known genetic diversity within *F. oxysporum* f. sp. *vasinfectum* (Table 1). Genetic relationships among different VCG were evaluated by phylogenetic analysis based on the *EF-1 α* , *PHO*, and *BT* genes comprising more than 5.6 kb of genomic sequence (Fig. 3). In all, 17 VCG, a few heterokaryon self-incompatible isolates, a few single-member VCG, and 23 sequence types in five lineage groups were observed within *F. oxysporum* from cotton (Fig. 3; Table 1). In most cases, a single VCG corresponded to a single sequence type, except one sequence type corresponded to three VCG (VCG0116, -01115, and -01116).

Reference isolates of *F. oxysporum* f. sp. *vasinfectum* races 1, 2, 3, 4, 6, 7, and 8; Australian VCG0111; and 11 additional VCG, as well as sequenced isolates of other *F. oxysporum* formae speciales and other *Fusarium* spp. (Broad Institute), were used to test specificity of the *Tfo1* insertion. Isolates representing other fungal species commonly associated with cotton were also tested. In total, 120 fungal strains were tested to validate the specificity of detection method (Table 1).

Multiplex PCR system differentiates between *F. oxysporum* f. sp. *vasinfectum* Cal race 4 isolates and other *F. oxysporum* f. sp. *vasinfectum* and fungal species. Twenty-four *F. oxysporum* f. sp. *vasinfectum* isolates from California cotton fields, consisting of

CA-9	348	GGCCGATATTGTCGGTCGTA	AGAGGATGTATGGTCTGGAG	CTTATTGTCATCATCTTTGC	GACTCTTGCCAGGCCCTGA	CTGCCGGTTCGCCTTCCACC
Race 4	348	GGCCGATATTGTCGGTCGTA	AGAGGATGTATGGTCTGGAG	CTTATTGTCATCATCTTTGC	GACTCTTGCCAGGCCCTGA	CTGCCGGTTCGCCTTCCACC
Race 7	348	GGCCGATATTGTCGGTCGTA	AGAGGATGTATGGTCTGGAG	CTTATTGTCATCATCTTTGC	GACTCTTGCCAGGCCCTGA	CTGCCGGTTCGCCTTCCACC
CA-9	448	TCGCTTGTTGGTCTT	<u>CAGTG TGCCATCAATCAAGTTACT</u>	TGGCTTGGTTGGTTGTGATT	GGTTGATTACGTAACAACCA	ACCAAAGTAAGGCTCCTGT
Race 4	448	TCGCTTGTTGGTCTT	-----	-----	-----	-----
Race 7	448	TCGCTTGTTGGTCTT	-----	-----	-----	-----
CA-9	86	TCAACCAAGCAAAGCAACCT	TTTTCCTTTACTCGGCTCTA	CTTGATTACTCGACAGAA	TGCCGAATGAGGCATAGCGC	GATGGCTTTGACATATCTAC
Race 4	-----	-----	-----	-----	-----	-----
Race 7	-----	-----	-----	-----	-----	-----
CA-9	186	GCGCTCTGATTGGCTGGGTA	TTCCGCATCCACCTGCGAA	AATTCTCGCCGAGTTTCTTA	AGAGAAGGTACCTGACGATT	GCGCCAAGCTCGAAGCCAAA
Race 4	-----	-----	-----	-----	-----	-----
Race 7	-----	-----	-----	-----	-----	-----
CA-9	286	<u>TATG</u> TCTGCACATTCACTT	TTCCGCCACATAGAAGGATC	AGGA/	/ACGG	AATCGCCTCAAGTCAGATAC
Race 4	-----	-----	-----	-----	-----	-----
Race 7	-----	-----	-----	-----	-----	-----
CA-9	2586	AAAAGCACCTGCTTCGGACC	GGCATGTTACCAATAGAAATT	GCCCCGATTGGGAATAGTAGT	CAAGTAATATCAACCAAGTA	AGGACTTCTTGTAAGCAACC
Race 4	-----	-----	-----	-----	-----	-----
Race 7	-----	-----	-----	-----	-----	-----
CA-9	2686	AAGTAAAGTAAAGGGCTCTA	GTCAACCAAGCCGAGTAAGG	GGGTCAAATTTTGGTTTACT	TGGTTGATGGACACACTGTT	<u>GGTCTT</u> [*] TATCATCTTCTGGCG
Race 4	463	-----	-----	-----	-----	-----ATCATCTTCTGGCG
Race 7	463	-----	-----	-----	-----	-----ATCATCTTCTGGCG
CA-9	477	TGTAGTCATGGGTGTTGGTA	TTGGTGGTGATTATCCCTT	TCTTCCATTATTACCTCCGA	GTAAGTTCATGTACACTCAA	GGCGATCATATCCAGGTTGA
Race 4	477	TGTAGTCATGGGTGTTGGTA	TTGGTGGTGATTATCCCTT	TCTTCCATTATTACCTCCGA	GTAAGTTCATGTACACTCAA	GGCGATCATATCCAGGTTGA
Race 7	477	TGTAGTCATGGGTGTTGGTA	TTGGTGGTGATTATCCCTT	TCTTCCATTATTACCTCCGA	GTAAGTTCATGTACACTCAA	GGCGATCATATCCAGGTTGA

Fig. 1. Sequence alignment of the phosphate: H⁺ symporter gene (*PHO*) of *Fusarium oxysporum* f. sp. *vasinfectum* isolates. Isolate CA-9 contains a *Tfo1* transposon insertion not found in reference isolates of race 4 from India (ATCC16613) or race 7 from China (*F. oxysporum* f. sp. *vasinfectum* NRRL25433). Boxed in is the conserved *hAT* transposon family's 15-bp terminal inverted repeat sequence while the 8-bp target site duplication (TSD) motif is in bold. Overlined nucleotides represent characteristic subterminal repeat regions. Reading frame of *Tfo1* is marked by the boxed-in and grayed-out start codon while the stop codon is grayed out. Asterisks (*) denote the beginning, recalibrated as base 1, and end of the *Tfo1* insertion, including the 8-bp 3' TSD generated by the transposon. Part (2.2 kb) of the *Tfo1* sequences have been omitted in the figure for spacing purposes.

Cal races 1, 3, 4, and 8, were assayed. Of these, 16 of 17 isolates previously identified as Cal race 4 produced the 583-bp amplicon characteristic of the *PHO-Tfo1* insertion (Fig. 2C; Table 1); isolate MD312 produced a 396-bp band. Isolate MD312 had moderate virulence in soil-infestation assays compared with the 16 isolates with

Fig. 2. Detection of Cal race 4 isolates of *Fusarium oxysporum* f. sp. *vasinfectum* using multiplex polymerase chain reaction (PCR). Cal race 4 strains produce a 583-bp amplicon while other *F. oxysporum* f. sp. *vasinfectum* isolates produce a 396-bp band based on *Tfo1* insertion status of the phosphate: H⁺ symporter (*PHO*) gene. **A**, Left panel: detection of *Tfo1* insertion in the *PHO* gene, where a 583-bp band is produced when an insertion is present (lane 1, Cal race 4 isolate CA-14) and a 396-bp band is produced when an intact *PHO* is present (lane 2, India race 4 reference isolate ATCC16613). Right panel: target location of PCR primers used in the multiplex. **B**, *F. oxysporum* f. sp. *vasinfectum* race isolates (left to right: race 1, ATCC16421; race 2, ATCC16611; race 3, ATCC16612; race 4, ATCC16613; race 6, ATCC36198; race 7, NRRL25433; and race 8, NRRL31665; Cal race 4, CA-9), analyzed with the multiplex system. **C**, Detection of *PHO-Tfo1* in California *F. oxysporum* f. sp. *vasinfectum* isolates representing diverse races: lane 1 (CA-1A) is an isolate with a race 8 sequence type; lane 2 (CA-1B) is a race 8 isolate; lanes 3 and 5 (CA-3 and CA-11, respectively) are isolates with DNA sequences similar to the race 3 reference isolate; lane 4 (CA-10) is a VCG01117A isolate with DNA sequences similar to races 6 reference isolate; and lanes 6 to 12 (CA-12, CA-14A, CA-S9, MD305, MD306, MD307, and CA-9, respectively) are Cal race 4 isolates. **D**, *F. oxysporum* isolates of diverse sequence types and vegetative compatibility groups (VCG) collected outside California: lanes 1, 3, 4, and 8 (CDR88, CDR538, CDR978, and Auseed5, respectively) are isolates with DNA sequences similar to those of the race 3 reference isolate; lanes 6, 7, and 10 (AL-KL11, MS-GL10, and AL-KL1, respectively) are isolates with a translation elongation factor 1 α DNA sequence, within limited stretches examined at an earlier study, identical to those of race 4 and which have been called "race 4-like" isolates; lanes 2 and 9 (CDR238 and Fov218, respectively) are race 1, 2, 6 lineage isolates; lane 5 (CDR943) is an isolate with DNA sequences similar to those of race 8 reference isolate; lane 11 (CA-9) is a Cal race 4 isolate; lane 12 is a negative control without a DNA template; and lane L is a 1 kb-plus DNA ladder.

the *PHO-Tfo1* insertion, which displayed a range of virulence. The remaining seven isolates of diverse race, sequence type, and VCG from California produced the 396-bp amplicon, denoting the lack of the *Tfo1* insertion. Similarly, 71 *F. oxysporum* isolates from cotton, including race 4 and 7 isolates, from fields outside of California produced only the 396-bp amplicon (Fig. 2D; Table 1). Moreover, all isolates from the seven named *F. oxysporum* f. sp. *vasinfectum* races (Fig. 2B; Table 1), seven nonpathogenic *F. oxysporum* isolates recovered from diseased cotton tissues or soils in the rhizosphere of cotton, eight isolates from seven other *F. oxysporum* formae speciales, one clinical isolate, and one biocontrol isolate of *F. oxysporum* produced only the 396-bp band (Table 1). Additionally, when assaying other *Fusarium* spp. (*F. verticillioides* 7600, *F. graminearum* PH-1, and *F. solani*) and other common soilborne fungal species, only *F. verticillioides* produced the 396-bp band and was negative for the *Tfo1* insertion band. No product was amplified from all other species when our primers were tested in the multiplex or two-primer PCR system (Table 1).

In addition to specificity, the sensitivity of the detection system was evaluated by assaying Cal race 4 isolate CA-9 and the race 4 reference isolate DNA. Testing concentrations ranging from 2 ng/ μ l to 600 fg/ μ l revealed a detection limit of 5 pg/ μ l for the *Tfo1* insert and 40 pg/ μ l for the undisrupted *PHO*, respectively, for the two isolates.

Diagnostic system provides reliable detection of Cal race 4 isolates in planta. The multiplex system was tested in planta with total DNA (fungal and plant DNA) extracted from infected and mock-inoculated plants 6 weeks after inoculation with CA-9 (Fig. 4A). The system was able to detect and amplify the 583-bp *Tfo1* insertion event in the extracted DNA at concentrations ranging from 800 to 12.5 ng of total DNA per 25- μ l reaction medium prepared from plants given a disease score of 2, or down to 25 ng from plants given a disease score of 1 (Fig. 4B). In order to determine the detection limits with fungal DNA in the presence of a large excess of plant total DNA, infected plant total DNA was mixed with uninfected plant DNA in 1:0, 1:1, 1:9, 1:24, 1:49, and 1:99 ratios. These mixed DNA samples were then used as template at a concentration of 50 ng of DNA per 25- μ l PCR assay. The system was able to amplify the 583-bp band down to a 1:49 dilution for infected plants with a disease score of 2. For less severely infected plants, with a disease score of 1, the band was discernible when the sample was diluted in a 1:9 ratio (Fig. 4C). Uninfected plant DNA did not amplify a band when tested with the multiplex system (Fig. 4B). The suitability of the plant sample's total DNA for PCR was independently confirmed using primers CelA1-F and CelA1-R targeting cotton's cellulose synthase A1 gene (Fig. 4D).

Cotton plants started to show symptoms about 3 weeks after inoculation with Cal race 4 isolate CA-9 (Fig. 4E). Initial symptoms often were severe but some plants did partially recover. Colonization by the pathogen in the 3-week-old infected plants with a disease severity rating of 3 was much less extensive than in the 6-week-old infected plants with a disease severity rating of 2 (Fig. 4E versus A), which was also reflected in the weaker *PHO-Tfo1* detection band intensity in the former plant (Fig. 4F versus B). CA-9 can also be detected in plants showing no external symptoms (Fig. 4E and F).

Discussion

We have serendipitously discovered that an *Ac*-like *Tfo1* transposon is present within the *PHO* gene of Cal race 4 isolate CA-9. Further screening revealed that this insertion is present in 15 of 16 other *F. oxysporum* f. sp. *vasinfectum* Cal race 4 isolates tested (Table 1). The exception is the California isolate MD312, which shares identical sequences with the India race 4 reference isolate and a China race 7 isolate in the three sequenced nuclear genes. The other 16 Cal race 4 isolates also share identical sequences with these isolates, except for the *Tfo1* insertion in the *PHO* gene. All four of the India race 4 reference isolates, all Cal race 4 isolates (including MD312), and the two China race 7 isolates belonged to VCG0114 (Table 1). Thus, all of these isolates were apparently derived from a single clonal lineage yet display distinct differences in pathogenicity.

The *PHO* gene is involved in the transport of inorganic phosphate across plasma membranes (Persson et al. 1999). Fragments of this gene have been widely used for *Fusarium* phylogenetic analyses (Kim et al. 2005; Liu et al. 2011; Skovgaard et al. 2001) as well as a species-specific marker because of its high degree of polymorphism (Persson et al. 1999; Stakheev et al. 2013). Previous studies analyzing the *PHO* gene sequence of CA-9 and other Cal Race 4 isolates were not able to detect this insertion due to the preferred *PHO*

fragment used for phylogenetic studies. Kim et al. (2005) amplified a 975-bp fragment of *PHO* and then used 513 bp of this fragment for their phylogenetic study. Their amplified fragment is located at base 466 of the *PHO* gene, just 4 bases downstream of the 3' target site duplication of the *Tfo1* insertion reported in this study.

The *Tfo1* insertional event in *PHO* of Cal race 4 was not found in any of the other *F. oxysporum* f. sp. *vasinfectum* isolates tested, which belong to 22 other sequence types and 23 other VCG (Fig. 3)

Fig. 3. Phylogenetic tree of diverse *Fusarium oxysporum* isolates. Tree constructed by the neighbor-joining method based on the concatenated sequences of translation elongation factor 1 α (1,831-bp aligned length), phosphate: H⁺ symporter (*PHO*; 1,945-bp aligned length), and β -tubulin (1,892-bp aligned length) from *F. oxysporum* f. sp. *vasinfectum* isolates representing known races, most vegetative compatibility groups (VCG), and sequence types. The *Tfo1* insertion sequence was coded as a single character in the *PHO* sequence. Sequences of a number of *F. oxysporum* formae speciales (*pisi* HDV247, *conglutinans* PHW808, *raphani* PHW815, *vasinfectum* NRRL25443, *lycopersici* 4287, *melonis* NRRL26406, *lycopersici* MN25, *radicis-lycopersici* CL57, and *cubense* II5), a clinical *F. oxysporum* isolate (FOSC 3-a), an *F. oxysporum* biocontrol agent (Fo47), and an *F. verticillioides* isolate (Fv7600), whose genome sequences are available from Broad Institute, were also included in the analysis as references. Sequences of *Fusarium* sp. isolate *AuSeed1* (Liu et al. 2011) was also included to root the tree. Distances were determined by Kimura's two-parameter model. Bootstrap frequencies from 1,000 replications are noted next to the branches. The scale is equivalent to 5 nucleotide substitutions per 1,000 bp.

representing all the known genetic diversity within *F. oxysporum* f. sp. *vasinfectum*. Moreover, no insertion at this locus was found among any of the 11 *F. oxysporum* genomes, or the *F. verticillioides* and *F. graminearum* genomes available from Broad Institute. Therefore, this unique insertion event provides a platform to develop a race-specific detection method for *F. oxysporum* f. sp. *vasinfectum* Cal race 4 isolates. The detection method described herein relies on three sequence-specific primers designed to simultaneously detect Cal race 4 and other *F. oxysporum* f. sp. *vasinfectum* by amplifying the *PHO-Tfo1* junction (FovP-F and FovT-R, 583-bp amplicon) or a fragment of the *F. oxysporum* *PHO* gene (FovP-F and FovP-R, 396-bp amplicon), respectively, in a duplex manner. This method amplified either the 583-bp band or the 396-bp band from all of the 107 *F. oxysporum* and 1 *F. verticillioides* isolates tested. The FovP-F and FovP-R pair positively identified all *F. oxysporum* f. sp. *vasinfectum* isolates other than Cal race 4 which contained the *Tfo1* insertion. The FovP-F and FovT-R pair is highly specific for the Cal race 4 isolates. The remaining 12 fungal isolates, including more distantly related *Fusarium* spp. and other soil-inhabiting fungal species, did not yield a product when tested with our primers. This failure was due to the specificity built into the primer's sequence for the *F. oxysporum* and closely related isolates. We used *EF-1 α* primers to produce an amplicon (data not shown) and verify the amenability of the fungal DNA used.

First characterized in *F. oxysporum* f. sp. *langenae*, *Tfo1* (transposon of *F. oxysporum*) is a transposable element, 2,763 bp long, encoding a 777-amino-acid transposase (Okuda et al. 1998). The element belongs to the *hAT* family of class II transposons (DNA transposon) because it contains common *hAT* structural characteristics such as 15-bp terminal inverted repeats, 8-bp target site duplications,

and a variable number (nine in *F. oxysporum* f. sp. *langenae*) of subterminal direct repeats (Fig. 1) (Kempken and Windhofer 2001; Okuda et al. 1998). The *Tfo1* element used in this detection study shares all of these features and greater than 99% sequence identity to the one previously reported.

Evidence suggests that *Tfo1* is a potentially active transposon (Okuda et al. 1998). Though there are some concerns on the position stability of active DNA transposons in a given genome due to its cut-and-paste transposition mechanism (Lievens et al. 2008), they are still used for pathogen detection. These include transposons *Fot1*, *Fot3*, *Hop*, and *Impala* in *Fusarium* spp. (Daboussi and Capy 2003). Thus, PCR amplification of the unique *Fot1* insertion junction has been used to specifically distinguish the date palm pathogen *F. oxysporum* f. sp. *albedinis* from other formae speciales (Fernandez et al. 1998). Furthermore, the *Fot1* and *Impala* insertion junctions were used to discriminate among different races within the carnation wilt pathogen *F. oxysporum* f. sp. *dianthi* (Chiocchetti et al. 1999). More recently, Suga et al. (2013) identified both DNA transposons and retrotransposons *Fot3*, *Foxy*, *Han*, *Hop*, *Hornet1*, *Impala*, and *Skippy* among isolates of *F. oxysporum* f. sp. *fragariae* and utilized the genomic region between *Han* and *Skippy* to distinguish the strawberry pathogen from other nonpathogenic *Fusarium* strains and five other formae speciales of *F. oxysporum*.

Retrotransposons (class I) transpose via a copy-and-paste mechanism; therefore, they are considered relatively more stable in the genome compared with DNA transposons (class II). The retroelements and techniques based on the retroelements, such as interretrotransposon sequence-characterized amplification region, have also been used to differentiate and detect a wide array of *F. oxysporum* isolates at either forma speciales or races levels for monophyletic groups

Fig. 4. Detection of *Fusarium oxysporum* f. sp. *vasinfectum* Cal race 4 in infected cotton plants with the multiplex polymerase chain reaction (PCR). **A**, Representative 6-week-old cotton plants used in this study: D1 (disease index 1 on a 0-to-4 scale, with 0 no disease and 4 plant dead) and D2 (disease index 2) are plants grown in soil drenched with CA-9 conidia ($5 \text{ ml of } 10^6 \text{ conidia ml}^{-1}$) and exhibit *Fusarium* wilt disease symptoms, while ND is a noninfected negative control plant. Total plant DNA was extracted from stem tissue (1 cm long) just above ground level. **B**, DNA from *F. oxysporum* f. sp. *vasinfectum*-infected plants at concentrations ranging from 800 to 12.5 ng per 25- μl reaction assayed with the PCR multiplex system. D1 in upper panel, D2 in lower panel. **C**, Phosphate: H^+ symporter (*PHO*)-*Tfo1* detection in total infected plant DNA diluted to the indicated ratios with noninoculated ND plant DNA. D1 in upper panel, D2 in lower panel. **D**, DNA amenability of total plant DNA from D1 (lane 1), D2 (lane 2), and ND (lane 3) plants by PCR amplification of the plant cellulose synthase A1 gene. **E**, Representative 3-week-old CA-9-infected cotton plants displaying no disease symptoms (D0) and displaying disease score of 3 (D3). **F**, *F. oxysporum* f. sp. *vasinfectum*-infected plant DNA at concentrations of 200 to 50 ng per 25- μl reaction assayed with the PCR multiplex system. D0 in upper panel, D3 in lower panel. For C, D, and F, CA-9 DNA from fungal culture was used as positive control whereas, for B, C, D, and F, ND DNA was used as a plant negative control. DNA from controls was used at 50 ng per reaction. X represents a reaction-negative (no DNA added) control while L represents a 1-kb-plus DNA ladder.

within respective levels (Lievens et al. 2008). However, retrotransposon stability may still be affected by the propensity of transposon elements to insert into existing elements, forming nested structures (Hua-Van et al. 2000). Because most transposons in *F. oxysporum* are DNA transposons (Dufresne et al. 2011; Hua-Van et al. 2000), they provide more target options compared with retroelements for use in pathogen differentiation in this species complex.

The developed method is also applicable for the in planta detection of Cal race 4 isolates with high specificity and sensitivity. The specificity of the assay was retained even at high concentrations of plant DNA. The sensitivity of detection was dependent on the disease severity as well as the age of the infected plants. The detection limit is lower for the more severely infected plants (Fig. 4B and F). Likewise, the 6-week-old infected plants had a lower detection limit than the 3-week-old infected plants with similar disease ratings, indicating that less extensive colonization by the pathogen during early stages of the infection process can still cause severe wilting symptoms. The system is even capable of detecting the presence of the pathogen in the plants before any external symptoms appear (Fig. 4F). For pure fungal DNA from isolate CA-9 and the reference isolate of race 4, a detection limit of 5 pg/μl for the *TfoI* insertion junction and 40 pg/μl for the undisrupted *PHO*, respectively, have been determined.

In conclusion, a method was developed to distinguish the highly virulent *F. oxysporum* f. sp. *vasinfectum* Cal race 4 isolates from other *F. oxysporum* f. sp. *vasinfectum* genotypes, including the race 4 reference isolate from India and other *Fusarium* spp. and soilborne fungi. The method involved screening for the transposable element *TfoI* within the coding sequence of the *PHO* gene of *F. oxysporum* f. sp. *vasinfectum*. The multiplex PCR assay described here is reliable in detecting Cal race 4 isolates, with no cross reactivity found to date. Therefore, the method provides a specific and reliable tool for the identification and detection of *F. oxysporum* f. sp. *vasinfectum* Cal race 4 isolates.

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