RESEARCH LETTER

Effector gene screening allows unambiguous identification of *Fusarium oxysporum* f. sp. *lycopersici* races and discrimination from other *formae speciales*

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Abstract

During infection of tomato, the fungus *Fusarium oxysporum* f. sp. *lycopersici* secretes several unique proteins, called 'secreted in xylem' (Six) proteins, into the xylem sap. At least some of these proteins promote virulence towards tomato and among them, all predicted avirulence proteins that can trigger disease resistance in tomato have been found. In this study, a large, worldwide collection of *F. oxysporum* isolates was screened for the presence of seven *SIX* genes (*SIX1*–*SIX7*). The results convincingly show that identification of *F. oxysporum formae speciales* and races based on host-specific virulence genes can be very robust. *SIX1*, *SIX2*, *SIX3* and *SIX5* can be used for unambiguous identification of the *forma specialis lycopersici*. In addition, *SIX4* can be used for the identification of race 1 strains, while polymorphisms in *SIX3* can be exploited to differentiate race 2 from race 3 strains. For *SIX6* and *SIX7*, close homologs were found in a few other *formae speciales*, suggesting that these genes may play a more general role in pathogenicity. Host specificity may be determined by the unique *SIX* genes, possibly in combination with the absence of genes that trigger resistance in the host.

Introduction

Fusarium oxysporum Schlechtend:Fr is an asexual fungus that is common in soils worldwide. Collectively, *F. oxysporum* strains can cause wilt or root, bulb or foot rot in a wide variety of plant species, among which are several economically important crops (Gordon & Martyn, 1997). Individual strains of *F. oxysporum*, however, usually infect only one or a few host species. Pathogenic strains have therefore been grouped into host-specific forms called *formae speciales* (f. spp.), which are sometimes divided further into races based on cultivar specificity (Armstrong & Armstrong, 1981; Di Pietro *et al*., 2003; Michielse & Rep, 2009). *Fusarium oxysporum* strains have also been assigned to vegetative compatibility groups (VCGs) (Puhalla, 1985), which correspond to clonal lineages of the fungus (Correll, 1991; Koenig *et al*., 1997; Kistler *et al*., 1998; Katan & Katan, 1999). While a particular *forma specialis* may cause disease in a certain plant species, strains belonging to other *formae speciales* may have a harmless or even a beneficial relation to the same species and vice versa (Recorbet *et al*., 2003). Therefore, discrimination between strains pathogenic and nonpathogenic towards a specific crop is essential in order to prevent unnecessary disease control efforts.

Classically, identification of pathogenic *F. oxysporum* isolates is based on pathogenicity testing (Recorbet *et al*., 2003), which is time consuming and laborious. In addition, as presently over 70 *formae speciales* have been described, an enormous number of plant species and cultivars should be used for correct strain identification (Fravel *et al*., 2003). Therefore, attempts are increasingly being made to replace these methods with molecular identification techniques (Lievens *et al*., 2008). Unfortunately, molecular discrimination of *F. oxysporum* isolates is seriously complicated by the polyphyletic nature of many *formae speciales*, such that isolates belonging to different *formae speciales* may be more related than isolates belonging to the same *forma specialis* (Kistler, 1997; Lievens *et al*., 2008). Ideally, molecular identification of *F. oxysporum* strains is based on DNA sequences directly related to (host-specific) pathogenicity or nonpathogenicity (Recorbet *et al*., 2003; Lievens *et al*., 2008).

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In many cases, the ability of a fungus to infect particular plant species depends on specific genes encoding host-determining ‘virulence factors’ that distinguish virulent from avirulent strains. These include small secreted proteins, called effectors, and enzymes involved in the synthesis of host-specific toxins (van der Does & Rep, 2007). Recently, several *in planta* secreted proteins have been identified in *F. oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder and H.N. Hans. *Fusarium oxysporum* f. sp. *lycopersici* is the causal agent of Fusarium wilt in tomato and has been reported in at least 32 countries (Jones *et al*., 1991). Several polymorphic resistance genes have been identified in tomato that each confers resistance against a subset of *F. oxysporum* f. sp. *lycopersici* strains. These resistance genes include *I* (for immunity), *I-1*, *I-2* and *I-3* (Huang & Lindhout, 1997). Races are named historically according to the resistance gene that is effective against them: the *I* gene and the (unlinked) *I-1* gene are effective only against race 1; *I-2* confers resistance to race 2 (which overcomes *I* and *I-1*); and *I-3* confers resistance to race 3 (which overcomes *I*, *I-1* and *I-2*) (Rep *et al*., 2005). Race 1 was initially described in 1886 (Booth, 1971). Race 2 was first reported in 1945 in Ohio (Alexander & Tucker, 1945) and race 3 was originally observed in Australia in 1978 (Grattidge & O’Brien, 1982). Subsequently, the different races have been reported in tomato crops worldwide. The first *in planta* secreted protein that was identified in *F. oxysporum* f. sp. *lycopersici*, called ‘secreted in xylem 1’ (Six1), is a small cysteine-rich protein required for full virulence on tomato (Rep *et al*., 2005). In addition, recognition of the protein by tomato plants carrying the resistance gene *I-3* leads to disease resistance (Rep *et al*., 2004). Therefore, Six1 is also called Avr3 to indicate its gene-for-gene relationship with the *I-3* resistance gene. More recently, additional fungal proteins were identified from xylem sap of infected plants, encompassing the small secreted proteins Six2, Six3, Six4, Six5, Six6 and Six7, an arabinanase, an oxidoreductase and a serine protease (Houterman *et al*., 2007; van der Does *et al*., unpublished data). While the function of most of these small proteins is unknown so far, for two of them, an avirulence function has been established using gene knockout experiments. These are Six4 (Avr1), which is required for *I* and *I-1*-mediated resistance (Houterman *et al*., 2008), and Six3 (Avr2), which is required for *I-2*-mediated resistance (Houterman *et al*., 2009). In addition, Six4/Avr1 was found to suppress *I-2*- and *I-3*-mediated disease resistance (Houterman *et al*., 2008). Race 2 strains are thought to have arisen through loss of *AVR1* (*SIX4*) from race 1 strains, while race 3 strains appear to have evolved from race 2 through point mutations in *AVR2* (*SIX3*) (Houterman *et al*., 2009). The strong link between these *SIX* genes and pathogenicity towards tomato makes them excellent markers for host- and cultivar-specific pathogenicity.

In this study, we show the usefulness of effector genes for reliable identification of host-specific fungal pathogens. Particularly, we show that *F. oxysporum* f. sp. *lycopersici* and its races can be unambiguously identified based on the above-mentioned *SIX* genes. The robustness of this approach is underscored by the fact that, like many other *formae speciales*, *F. oxysporum* f. sp. *lycopersici* strains do not have a common ancestor within the *F. oxysporum* species complex (O’Donnell *et al*., 1998; van der Does *et al*., 2008).

Materials and methods

Fungal isolates

A worldwide collection of 270 *F. oxysporum* strains, obtained from diverse geographic origins, was assembled in this study (Table 1). One hundred and sixty-four strains isolated from tomato were used, encompassing 15 avirulent isolates, 75 *F. oxysporum* f. sp. *lycopersici* strains and 74 isolates belonging to *F. oxysporum* f. sp. *radicis-lycopersici*, representing most known VCGs of these *formae speciales* (Katan & Katan, 1999). While both *formae speciales* share tomato as the same host, they cause different symptoms: *F. oxysporum* f. sp. *lycopersici* causes wilt and *F. oxysporum* f. sp. *radicis-lycopersici* causes root and foot rot (Menzies *et al*., 1990). In addition, 106 *F. oxysporum* isolates of 14 other *formae speciales* were included in our study as well as seven isolates of three other *Fusarium* species (Table 1). For most of these isolates, pathogenicity, vegetative compatibility and genetic diversity have been assessed in previous studies (e.g. Katan *et al*., 1991; Marlatt *et al*., 1996; O’Donnell *et al*., 1998; Katan & Katan, 1999; Vakalounakis & Fragkiadakis, 1999; Baayen *et al*., 2000; Cai *et al*., 2003; Vakalounakis *et al*., 2004; Balmas *et al*., 2005; Kawabe *et al*., 2005; Lievens *et al*., 2007; van der Does *et al*., 2008). Isolates were grown on potato dextrose agar containing 0.1 $mg\,mL^{-1}$ streptomycin sulfate in the dark at 22 °C.

PCR analysis and sequencing

All isolates listed in Table 1 were subjected to PCR analysis using primers that were designed previously, amplifying *SIX1*, *SIX2* and *SIX3* (Rep *et al*., 2004; van der Does *et al*., 2008; Table 2). In addition, all isolates were screened using primers that were located just outside the respective ORFs of *SIX4*, *SIX5*, *SIX6* and *SIX7* (Table 2). In order to check DNA quality, PCR amplification was performed using the universal primers ITS5 and ITS4, which anneal to conserved regions of the 18S and 28S rRNA genes, respectively (White *et al*., 1990). Genomic DNA was extracted using the phenol–chloroform extraction method as described previously (Lievens *et al*., 2003) and the yield was determined spectrophotometrically. PCR amplification was carried out in a reaction volume of 20 μL, containing

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Table 1. *Fusarium oxysporum* strains used in this study

Isolate[*]	VCG[†]	Race[‡]	Geographic origin	Source[§]	PCR screen targeting the *SIX* genes[¶]						
					SIX1	*SIX2*	*SIX3*	*SIX4*	*SIX5*	*SIX6*	*SIX7*
F. oxysporum f. sp. *Lycopersici*											
218	0030	1	Spain	M.I.G.R.	+	+	+	+	+	+	+
D1	0030	1	France	L.D.	+‖	+	+	+‖	+	+	+
E179	0030-si	1	USA	C.H.B.	+‖	+	+	+	+	+	+
Fol1 (66044)	0030	1	Israel	T.K.	+	+	+	+	+	+	+
FOL-24L	0030	1	Israel	T.K.	+	+	+	+	+	+	+
Fol-650B	0030	1	Israel	T.K.	+‖	+	+	+	+	+	+
FOL-HH6M	0030	1	Israel	T.K.	+	+	+	+	+	+	+
Fol-R5-6/E172	0030-si	1	Wisconsin, USA	R.J.S.	+‖	+	+	+	+	+	+
IPO1530/B1	0030	1	The Netherlands	IPO-DLO	+‖	+‖	+‖	+‖	+‖	+‖	+‖
LSU-3	0030	1	Louisiana, USA	K.S.E.	+‖	+	+	+	+	+	+
WCS861/E240	0030	1	The Netherlands	N.A.M.V.S.	+‖	+	+	+‖	+	+	+
18947	0030	2	Australia	D.J.M.	+	+	+	−	+	+	+
281	0030	2	Spain	M.I.G.R.	+	+	+	−	+	+	+
4287	0030	2	Spain	M.I.G.R.	+	+	+	−	+	+	+
548	0030	2	Florida, USA	J.P.J.	+	+	+	−	+	+	+
BFOL-53	0030	2	Louisiana, USA	K.S.E.	+‖	+	+	−	+	+	+
BFOL-70	0030	2	Louisiana, USA	K.S.E.	+‖	+	+	−	+	+	+
D2	0030-si	2	France	L.D.	+‖	+‖	+‖	−	+‖	+‖	+‖
Fol-1295T (66047)	0030	2	Israel	T.K.	+	+	+	−	+	+	+
FOL-93H	0030	2	Israel	T.K.	+	+	+	−	+	+	+
FRC-0-1078	0030	2	Florida, USA	T.K.	+	+	+	−	+	+	+
WCS862/E241	0030	2	The Netherlands	N.A.M.V.S.	+‖	+‖	+‖	−	+‖	+‖	+‖
FOL-MM59	0030	2	Arkansas, USA	T.K.	+	+	+	−	+	+	+
FOL-MM66	0030	2	Arkansas, USA	T.K.	+	+	+	−	+	+	+
LSU-7	0030	2	Louisiana, USA	K.S.E.	+‖	+‖	+‖	−	+‖	+‖	+‖
14844 (M1943)	0030	3	Australia	D.J.M.	+‖	+	+‖	−	+	+	+
5397	0030	3	Florida, USA	J.P.J.	+	+‖	+‖	−	+‖	+‖	+‖
CA92/95	0030	3	California, USA	D.A.L.	+‖	+	+‖	−	+	+	+
Fol036	0030	3	Florida, USA	J.W.S.	+‖	+	+‖	−	+	+	+
IPO3	0030	3	The Netherlands	IPO-DLO	+‖	+	+‖	−	+	+	+
MX395	0030	1[‡]	Mexico	D.A.L.	+‖	+	+	+	+	+	+
NRRL 26037	0030	1	Florida, USA		+	+	+	−	+	+	+
FOL-295A	0030	1[‡]	Israel	T.K.	+	+	+	+	+	+	+
01109/3	0031	1	Belgium	T.K.	+	+	+	+	+	+	+
BFOL-51	0031	1	Louisiana, USA	K.S.E.	+‖	+‖	+‖	+‖	+‖	+‖	+‖
E175	0031	1	The Netherlands	D.M.E.	+‖	+‖	+‖	+‖	+‖	+‖	+‖
OSU-451	0031	2	Ohio, USA	K.S.E.	+‖	+‖	+‖	−	+‖	+‖	+‖
FOL-lyc 07038	0031	Unknown	Japan	T.K.	+	+	+	+	+	+	+
FRC-0-1113N	0031	1[‡]	New Hampshire, USA	T.K.	+	+	+	+	+	+	+

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Table 1. Continued.

Isolate[*]	VCG[†]	Race[‡]	Geographic origin	Source[§]	PCR screen targeting the *SIX* genes[¶]						
					SIX1	*SIX2*	*SIX3*	*SIX4*	*SIX5*	*SIX6*	*SIX7*
FRC-0-1113A	0031	1[‡]	New Hampshire, USA	T.K.	+	+	+	+	+	+	+
FOL-MM10	0033	3	Arkansas, USA	T.K.	+[‖]	+[‖]	+[‖]	–	+	+	+
FOL-MM25	0033	3	Arkansas, USA	T.K.	+	+	+[‖]	–	+	+	+
E79	003-sc	1	The Netherlands	IPO-DLO	+[‖]	+[‖]	+[‖]	+[‖]	+[‖]	+[‖]	+[‖]
C24/B2	003-sc	2	The Netherlands	IPO-DLO	+[‖]	+[‖]	+[‖]	–	+[‖]	+[‖]	+[‖]
48112	003-si	1	Spain	M.I.G.R.	+	+	+	+	+	+	+
626	003-si	1	Florida, USA	J.P.J.	+	+	+	+	+	+	+
E181	003-si	1	The Netherlands	D.M.E.	+[‖]	+	+	+	+	+	+
WCS801/E329	003-si	1	The Netherlands	D.M.E.	+[‖]	+[‖]	+[‖]	+[‖]	+[‖]	+[‖]	+[‖]
ATCC 417	Unknown	1	USA		+	+	+	+	+	+	+
CBS 412.90	Unknown	1	Israel		+	+	+	+	+	+	+
CBS 645.78	Unknown	2[‡]	Morocco		+	+	+	–	+	+	+
DSM 62059	Unknown	1	The Netherlands		+	+	+	+	+	+	+
EY-101	Unknown	1	Egypt	P.S.	+	+	+	+	+	+	+
FOL1	Unknown	1	Unknown	H.C.K.	+	+	+	+	+	+	+
MD-L3	Unknown	1	USA	P.S.	+	+	+	+	+	+	+
MD-S2	Unknown	1	USA	P.S.	+[‖]	+[‖]	+[‖]	+[‖]	+[‖]	+[‖]	+[‖]
ATCC 605	Unknown	2	USA		+	+	+	–	+	+	+
CBS 413.90	Unknown	2	Israel		+	+	+	–	+	+	+
CBS 414.90	Unknown	2	Israel		+	+	+	–	+	+	+
CBS 646.78	Unknown	1[‡]	Morocco		+	+	+	+	+	+	+
EY-102	Unknown	2	Egypt	P.S.	+	+	+	–	+	+	+
FOL2	Unknown	2	Unknown	H.C.K.	+	+	+	–	+	+	+
Fol-W841D (76535)	Unknown	2	Israel	T.K.	+	+	+	–	+	+	+
CBS 164.85	Unknown	1[‡]	The Netherlands		+	+	+	+[‖]	+	+	+
CBS 165.85	Unknown	1[‡]	The Netherlands		+	+	+	+	+[‖]	+[‖]	+[‖]
CBS 758.68	Unknown	1[‡]	The Netherlands		+	+	+	+	+	+	+
DSM 62338	Unknown	1[‡]	Italy		+	+	+	+	+	+	+
FOL 00/60309/1	Unknown	1[‡]	Unknown	F.V.	+	+	+	+	+	+	+
Fol045	Unknown	Unknown	Unknown		+	+	+	–	+	+	+
MUCL 19445	Unknown	Unknown	Unknown		+	+	+	+	+	+	+
NRRL 26034	Unknown	1[‡]	Unknown		+	+	+	+	+	+	+
NRRL 26200	Unknown	Unknown	Ohio, USA		+[‖]	+[‖]	+	–	+	+	+
NRRL 26202	Unknown	Unknown	Unknown		+	+	+	–	+	+	+
NRRL 26203	Unknown	Unknown	Italy		+[‖]	+[‖]	+	+	+	+	+
NRRL 26380[**]	Unknown	Unknown	Florida, USA		+[‖]	+[‖]	+	–	+	+	+
F. oxysporum f. sp. *radicis-lycopersici*											
ATCC 52429	0090 I		Canada		–	–	–	–	–	–	–
DP83	0090 I		Italy	Q.M.	–	–	–	–	–	–	–
FORL-19R	0090 I		France	T.K.	–	–	–	–	–	–	–

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Table 1. Continued.

Isolate*	VCG[†]	Race[‡]	Geographic origin	Source[§]	PCR screen targeting the *SIX* genes[¶]						
					SIX1	*SIX2*	*SIX3*	*SIX4*	*SIX5*	*SIX6*	*SIX7*
FORL-C405B	0090 I		Israel	T.K.	–	–	–	–	–	–	–
FORL-C709	0090 I		Israel	T.K.	–	–	–	–	–	–	–
FORL-DJV78	0090 I		Greece	T.K.	–	–	–	–	–	–	–
FRC-0-1090	0090 I		Canada	T.K.	–	–	–	–	–	–	–
HRS-SB153R	0090 I		Canada	T.K.	–	–	–	–	–	–	–
FORL-C1018F	0090 II		Israel	T.K.	–	–	–	–	–	–	–
FORL-C809L	0090 II		Israel	T.K.	–	–	–	–	–	–	–
DP95	0090 III		Italy	Q.M.	–	–	–	–	–	–	–
FORL-838H	0090 III		Israel	T.K.	–	–	–	–	–	–	–
FORL-C1058P	0090 III		Israel	T.K.	–	–	–	–	–	–	–
FORL-C696A	0090 III		Israel	T.K.	–	–	–	–	–	–	–
FORL-C710B	0090 III		Israel	T.K.	–	–	–	–	–	–	–
S7	0090 III		Italy	Q.M.	–	–	–	–	–	–	–
DP61	0091		Italy	Q.M.	–	–	–	–	–	–	–
ATCC 60095	0091 I		Canada		–	–	–	–	–	–	–
FORL-89-1511	0091 I		France	T.K.	–	–	–	–	–	–	–
FORL-C58M	0091 I		Israel	T.K.	–	–	–	–	–	–	–
FORL-GAR3	0091 I		Italy	T.K.	–	–	–	–	–	–	–
FORL-OSU374	0091 I		Ohio, USA	T.K.	–	–	–	–	–	–	–
FORL-Pt 473C	0091 I		USA	T.K.	–	–	–	–	–	–	–
FORL-Pt 473D	0091 I		USA	T.K.	–	–	–	–	–	–	–
FRC-0-1097 K	0091 I		Canada	T.K.	–	–	–	–	–	–	–
FU-87-1	0091 I		The Netherlands	T.K.	–	–	–	–	–	–	–
J-36	0091 I		Canada	T.K.	–	–	–	–	–	–	–
01157	0091 II		Belgium	T.K.	–	–	–	–	–	–	–
FORL-C1327A	0091 II		Israel	T.K.	–	–	–	–	–	–	–
FORL-C434	0091 II		Israel	T.K.	–	–	–	–	–	–	–
FORL-C734B	0091 II		Israel	T.K.	–	–	–	–	–	–	–
FORL-CRH673	0091 II		Israel	T.K.	–	–	–	–	–	–	–
FORL-UK9B	0091 II		UK	T.K.	–	–	–	–	–	–	–
HRS-SB082Q	0091 II		Canada	T.K.	–	–	–	–	–	–	–
FORL-C815A	0092		Israel	T.K.	–	–	–	–	–	–	–
DP37	0093		Italy	Q.M.	–	–	–	–	–	–	–
DP44	0093		Italy	Q.M.	–	–	–	–	–	–	–
FORL-C202	0093		Israel	T.K.	–	–	–	–	–	–	–
MUCL 39790	0094		Belgium		–	–	–	–	–	–	–
MUCL 39791	0094		Belgium		–	–	–	–	–	–	–
MUCL 39792	0094		Belgium		–	–	–	–	–	–	–
MUCL 39793	0094		Belgium		–	–	–	–	–	–	–
MUCL 39794	0094		Belgium		–	–	–	–	–	–	–

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Table 1. Continued.

Isolate*	VCG[†]	Race[‡]	Geographic origin	Source[§]	PCR screen targeting the *SIX* genes[¶]						
					SIX1	*SIX2*	*SIX3*	*SIX4*	*SIX5*	*SIX6*	*SIX7*
01150	0094 I		Belgium	T.K.	–	–	–	–	–	–	–
FORL-UK3Q	0094 I		UK	T.K.	–	–	–	–	–	–	–
FORL-FL418	0094 II		Korea	T.K.	–	–	–	–	–	–	–
DP282	0096		Italy	Q.M.	–	–	–	–	–	–	–
FORL-C622A	0096		Israel	T.K.	–	–	–	–	–	–	–
FORL-C623	0096		Israel	T.K.	–	–	–	–	–	–	–
FORL-C624A	0096		Israel	T.K.	–	–	–	–	–	–	–
FORL-C651	0096		Israel	T.K.	–	–	–	–	–	–	–
PB9	0098		Florida, USA	Q.M.	–	–	–	–	–	–	–
01090/B	Unknown		Unknown	H.C.K.	–	–	–	–	–	–	–
41	Unknown		Canada	Z.K.P.	–	–	–	–	–	–	–
42	Unknown		Canada	Z.K.P.	–	–	–	–	–	–	–
43	Unknown		Canada	Z.K.P.	–	–	–	–	–	–	–
46	Unknown		Canada	Z.K.P.	–	–	–	–	–	–	–
CBS 101587	Unknown		Unknown		–	–	–	–	–	–	–
CBS 872.95	Unknown		Israel		–	–	–	–	–	–	–
CBS 873.95	Unknown		Israel		–	–	–	–	–	–	–
CBS 874.95	Unknown		Israel		–	–	–	–	–	–	–
FORL 00/60309/2	Unknown		Unknown	F.V.	–	–	–	–	–	–	–
MUCL 38936	Unknown		Belgium		–	–	–	–	–	–	–
MUCL 39788	Unknown		Belgium		–	–	–	–	–	–	–
MUCL 39789	Unknown		Belgium		–	–	–	–	–	–	–
MUCL 39795	Unknown		Belgium		–	–	–	–	–	–	–
MUCL 39796	Unknown		Belgium		–	–	–	–	–	–	–
MUCL 39797	Unknown		Belgium		–	–	–	–	–	–	–
MUCL 39798	Unknown		Belgium		–	–	–	–	–	–	–
MUCL 39799	Unknown		Belgium		–	–	–	–	–	–	–
MUCL 39800	Unknown		Belgium		–	–	–	–	–	–	–
NRRL 26033	Unknown		Florida, USA		–	–	–	–	–	–	–
NRRL 26379	Unknown		Florida, USA		–	–	–	–	–	–	–
NRRL 26381	Unknown		Florida, USA		–	–	–	–	–	–	–
Nonpathogenic isolates from tomato											
CBS 249.52[††]	Unknown		Unknown		–	–	–	–	–	–	–
E184[††]	Unknown		Bulgaria		–	–	–	–	–	–	–
MUCL 14159[††]	Unknown		Unknown		–	–	–	–	–	–	–
NRRL 22544[††]	Unknown		Germany		–	–	–	–	–	–	–
ST FO 5	Unknown		Belgium	B.L.	–	–	–	–	–	–	–
ST FO 6	Unknown		Belgium	B.L.	–	–	–	–	–	–	–
ST FO 8	Unknown		Belgium	B.L.	–	–	–	–	–	–	–
ST FO 9	Unknown		Belgium	B.L.	–	–	–	–	–	–	–

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Table 1. Continued.

Isolate*	VCG†	Race‡	Geographic origin	Source§	PCR screen targeting the *SIX* genes¶						
					SIX1	*SIX2*	*SIX3*	*SIX4*	*SIX5*	*SIX6*	*SIX7*
ST FO 10	Unknown		Belgium	B.L.	–	–	–	–	–	–	–
ST FO 11	Unknown		Belgium	B.L.	–	–	–	–	–	–	–
ST FO 12	Unknown		Belgium	B.L.	–	–	–	–	–	–	–
ST FO 13	Unknown		Belgium	B.L.	–	–	–	–	–	–	–
ST FO 14	Unknown		Belgium	B.L.	–	–	–	–	–	–	–
ST FO 15	Unknown		Belgium	B.L.	–	–	–	–	–	–	–
ST FO 16	Unknown		Belgium	B.L.	–	–	–	–	–	–	–
F. oxysporum f. sp. *asparagi*											
NRRL 28973	1001		USA		–	–	–	–	–	–	–
NRRL 28362	1002		USA		–	–	–	–	–	–	–
NRRL 28379	1008		USA		–	–	–	–	–	–	–
F. oxysporum f. sp. *conglutinans*											
81-4	Unknown		Unknown	H.C.K.	–	–	–	–	–	–	–
F. oxysporum f. sp. *cubense*											
NRRL 25603	0120		Australia		–	–	–	–	–	–	–
NRRL 26029	01210		Florida, USA		–	–	–	–	–	–	–
NRRL 25609	01214		Malawi		–	–	–	–	–	–	–
F. oxysporum f. sp. *cucumerinum*											
Afu-50(B)	0180		Crete	D.J.V.	–	–	–	–	–	–	–
Afu-52	0180		Crete	D.J.V.	–	–	–	–	–	–	–
Afu-57(B)	0180		Crete	D.J.V.	–	–	–	–	–	–	–
ATCC 16416	0180		Florida, USA		–	–	–	–	–	–	–
ATCC 201950	0180		Florida, USA		–	–	–	–	–	–	–
ATCC 36330	0180		Israel		–	–	–	–	–	–	–
FOCU-16F	0180		Israel	T.K.	–	–	–	–	–	–	–
FOCU-17W	0180		Israel	T.K.	–	–	–	–	–	–	–
FOCU-22P	0180		Israel	T.K.	–	–	–	–	–	–	–
FOCU-26E	0180		Israel	T.K.	–	–	–	–	–	–	–
FOCU-33N	0180		Israel	T.K.	–	–	–	–	–	–	–
FOCU-39E	0180		Israel	T.K.	–	–	–	–	–	–	–
FOCU-45K	0180		Israel	T.K.	–	–	–	–	–	–	–
FOCU-48F	0180		Israel	T.K.	–	–	–	–	–	–	–
FOCU-707E	0180		Israel	T.K.	–	–	–	–	–	–	–
FOCU-CM1C	0180		Israel	T.K.	–	–	–	–	–	–	–
Cu:4-1	0181		Japan	D.J.V.	–	–	–	–	–	–	–
NETH 10782(B)	0181		The Netherlands	D.J.V.	–	–	–	–	–	–	–
NETH 11179	0181		The Netherlands	D.J.V.	–	–	–	–	–	–	–
ATCC 36332	0182		Japan	D.J.V.	–	–	–	–	–	–	–
0018	0183		China	D.J.V.	–	–	–	–	–	–	–
Cu:5-0	0183		Japan	D.J.V.	–	–	–	–	–	–	–

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Table 1. Continued.

Isolate*	VCG[†]	Race[‡]	Geographic origin	Source[§]	PCR screen targeting the *SIX* genes[¶]						
					SIX1	*SIX2*	*SIX3*	*SIX4*	*SIX5*	*SIX6*	*SIX7*
9906-2	0184		China	D.J.V.	–	–	–	–	–	–	–
9906-3	0184		China	D.J.V.	–	–	–	–	–	–	–
9909-2	0185		China	D.J.V.	–	–	–	–	–	–	–
Tf-213	0185		China	D.J.V.	–	–	–	–	–	–	–
9901-2	0186		China	D.J.V.	–	–	–	–	–	–	–
9903-1	0186		China	D.J.V.	–	–	–	–	–	–	–
9903-2	0186		China	D.J.V.	–	–	–	–	–	–	–
9904-1	0186		China	D.J.V.	–	–	–	–	–	–	–
9909-3	0186		China	D.J.V.	–	–	–	–	–	–	–
0016	0187		China	D.J.V.	–	–	–	–	–	–	–
0017	0187		China	D.J.V.	–	–	–	–	–	–	–
0020	0187		China	D.J.V.	–	–	–	–	–	–	–
10196	Unknown		Unknown	A.C.M.C.	–	–	–	–	–	–	–
ATCC 42352	Unknown		Japan		–	–	–	–	–	–	–
ATCC 42357	Unknown		Japan		–	–	–	–	–	–	–
D1-RL	Unknown		Taiwan	Y.T.W.	–	–	–	–	–	–	–
D4-33	Unknown		Taiwan	Y.T.W.	–	–	–	–	–	–	–
DSM 62313	Unknown		Germany		–	–	–	–	–	–	–
FOC 00/0092/1	Unknown		Unknown	F.V.	–	–	–	–	–	–	–
MAFF 103054	Unknown		Japan		–	–	–	–	–	–	–
MAFF 305116	Unknown		Japan		–	–	–	–	–	–	–
MAFF 305117	Unknown		Japan		–	–	–	–	–	–	–
MAFF 727508	Unknown		Japan		–	–	–	–	–	–	–
MAFF 744004	Unknown		Japan		–	–	–	–	–	–	–
MAFF 744005	Unknown		Japan		–	–	–	–	–	–	–
NRRL 26437	Unknown		South Carolina, USA		–	–	–	–	–	–	–
F. oxysporum f. sp. *dianthi*											
NRRL 26147	0020		USA		–	–	–	–	–	–	–
NRRL 26960	0025		The Netherlands		–	–	–	–	–	–	–
F. oxysporum f. sp. *gladioli*											
NRRL 28914	0340		The Netherlands		–	–	–	–	–	–	–
NRRL 26993	0343		Italy		–	–	–	–	–	–	–
NRRL 26990	0345		The Netherlands		–	–	–	–	–	–	–
F. oxysporum f. sp. *lilii*											
NRRL 26955	0190		The Netherlands		–	–	–	–	–	–	+[‖]
NRRL 28395	0190		Italy		–	–	–	–	–	–	+[‖]
F. oxysporum f. sp. *luffae*											
Fol-114	Unknown		Taiwan	Y.T.W.	–	–	–	–	–	–	–
Fol-167	Unknown		Taiwan	Y.T.W.	–	–	–	–	–	–	–

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Table 1. Continued.

Isolate*	VCG[†]	Race[‡]	Geographic origin	Source[§]	PCR screen targeting the *SIX* genes[¶]						
					SIX1	*SIX2*	*SIX3*	*SIX4*	*SIX5*	*SIX6*	*SIX7*
F. oxysporum f. sp. *melonis*											
NRRL 26406	0136		Mexico		–	–	–	–	–	+[‖]	–
F. oxysporum f. sp. *niveum*											
CBS 187.60	Unknown		Germany		–	–	–	–	–	–	–
CBS 418.90	Unknown		Israel		–	–	–	–	–	–	–
CBS 419.90	Unknown		Israel		–	–	–	–	–	–	–
F. oxysporum f. sp. *opuntiarum*											
NRRL 28363	0450		The Netherlands		–	–	–	–	–	–	–
NRRL 28368	0451		The Netherlands		–	–	–	–	–	–	–
NRRL 28279	0454		Germany		–	–	–	–	–	–	–
F. oxysporum f. sp. *radicis-cucumerinum*											
Afu-11(A)	0260		Crete	D.J.V.	–	–	–	–	–	+	–
Afu-29(B)	0260		Crete	D.J.V.	–	–	–	–	–	–	–
Afu-3	0260		Crete	D.J.V.	–	–	–	–	–	+[‖]	–
Afu-33	0260		Crete	D.J.V.	–	–	–	–	–	–	–
Afu-44(B)	0260		Crete	D.J.V.	–	–	–	–	–	+[‖]	–
Afu-58	0260		Crete	D.J.V.	–	–	–	–	–		
Afu-68(A)	0260		Crete	D.J.V.	–	–	–	–	–	–	–
Afu-72	0260		Crete	D.J.V.	–	–	–	–	–	+	–
Afu-4(A)	0261		Crete	D.J.V.	–	–	–	–	–	–	–
AK-2	0261		Crete	D.J.V.	–	–	–	–	–	+[‖]	–
8	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
14	Unknown		Canada	Z.K.P.	–	–	–	–	–	–	–
16	Unknown		Canada	Z.K.P.	–	–	–	–	–	+[‖]	–
20	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
21A	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
22	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
24	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
28	Unknown		Canada	Z.K.P.	–	–	–	–	–	+[‖]	–
29	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
30	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
31	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
32	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
33	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
34	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
35	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
36	Unknown		Canada	Z.K.P.	–	–	–	–	–	+	–
38	Unknown		France	Z.K.P.	–	–	–	–	–	+	–
60B	Unknown		France	Z.K.P.	–	–	–	–	–	+	–
FORC 00/0092/2	Unknown		Unknown	F.V.	–	–	–	–	–	+[‖]	–

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Table 1. Continued.

Isolate*	VCG†	Race‡	Geographic origin	Source§	PCR screen targeting the *SIX* genes¶						
					SIX1	*SIX2*	*SIX3*	*SIX4*	*SIX5*	*SIX6*	*SIX7*
F. oxysporum f. sp. *Spinaciae*											
NRRL 26874	0330		Arkansas, USA		–	–	–	–	–	–	–
NRRL 26875	0331		Arkansas, USA		–	–	–	–	–	–	–
NRRL 26876	0332		Arkansas, USA		–	–	–	–	–	–	–
F. oxysporum f. sp. *tulipae*											
NRRL 22556	0230		Germany		–	–	–	–	–	–	–
NRRL 26954	0230		The Netherlands		–	–	–	–	–	–	–
NRRL 28974	0230		The Netherlands		–	–	–	–	–	–	–
F. graminearum											
PH1	–		Unknown	H.C.K.	–	–	–	–	–	–	–
F. javanicum											
CBS 410.62	–		The Netherlands		–	–	–	–	–	–	–
CBS 616.66	–		The Netherlands		–	–	–	–	–	–	–
F. solani											
MUCL 20259	–		Unknown		–	–	–	–	–	–	–
CBS 165.87	–		Denmark		–	–	–	–	–	–	–
CABI 17960	–		Brazil		–	–	–	–	–	–	–
S-66	–		Unknown	H.C.K.	–	–	–	–	–	–	–

*ATCC, American Type Culture Collection, Manassas, VA; CABI, Centre for Agriculture and Bioscience International, Surrey, UK; CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; MAFF, Genetic Resources Management Section, GenBank, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki, Japan; MUCL, Mycotèque de l'Université Catholique de Louvain, Louvain-la-Neuve, Belgium; NRRL, Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL.

†Kawabe *et al*. (2005) showed that the VCG 0030 tester isolate NRRL 26037 and some other isolates of VCG 0030 were weakly compatible with VCG 0032 tester isolate FOL-MM66. In addition, FOL-MM59 (VCG 0032) was found to be a bridging isolate that can also form heterokaryons with some members of VCG 0030 (Cai *et al*., 2003; Kawabe *et al*., 2005). sc, self-compatible; si, self-incompatible.

‡If known, race is mentioned for *F. oxysporum* f. sp. *lycopersici*. A pathogenicity test on a tomato line carrying the gene revealed that isolate CBS 645.78 was originally misidentified as race 1, while isolates CBS 646.78 and MX295 should be designated as race 1 isolates instead of race 2 and race 3, respectively. In addition, nine isolates of unknown race were tested and turned out to be race 1. These strains included CBS 164.85, CBS 165.85, CBS 758.68, DSM 62338, FOL 00/60309/1, FOL-295A, FRC-0-1113A, FRC-0-1113N and NRRL 26034.

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¶+, amplicon detected; –, no amplicon detected. A fragment of 992, 749, 608, 967, 667, 793 and 862 bp is generated for *SIX1*, *SIX2*, *SIX3*, *SIX4*, *SIX5*, *SIX6* and *SIX7*, respectively. As a check for DNA quality, all DNA samples were successfully subjected to PCR analysis using the universal primers ITS5 and ITS4, targeting the rRNA gene (White *et al*., 1990).

‖Sequence determined.

**Previously misidentified as *F. oxysporum* f. sp. *radicis-lycopersici*, recently classified as *F. oxysporum* f. sp. *lycopersici* (Kawabe *et al*., 2005; van der Does *et al*., 2008).

††Previously misidentified as *F. oxysporum* f. sp. *lycopersici*, recently determined as nonpathogenic to tomato (van der Does *et al*., 2008).

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Table 2. Primers used in this study

Code	Sequence (5′–3′)	Target	Amplicon length (bp)	Reference
P12-F2B	GTATCCCTCCGGATTTTGAGC	*SIX1*	992	van der Does *et al*. (2008)
P12-R1	AATAGAGCCTGCAAAGCATG	*SIX1*		Rep *et al*. (2004)
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	–
SIX4-R1	GCCGACCGAAAAACCCTAA	*SIX4*		–
SIX5-F1	ACACGCTCTACTACTCTTCA	*SIX5*	667	–
SIX5-R1	GAAAACCTCAACGCGGCAAA	*SIX5*		–
SIX6-F1	CTCTCCTGAACCATCAACTT	*SIX6*	793	–
SIX6-R1	CAAGACCAGGTGTAGGCATT	*SIX6*		–
SIX7-F1	CATCTTTTCGCCGACTTGGT	*SIX7*	862	–
SIX7-R1	CTTAGCACCCTTGAGTAACT	*SIX7*		–

0.15 mM of each dNTP, 0.5 μM of each primer, 1 × Titanium *Taq* DNA polymerase, 1 × Titanium *Taq* PCR buffer (Clontech Laboratories, Palo Alto, CA) and 5 ng genomic DNA. Thermal cycling conditions consisted of 2 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 59 °C and 45 s at 72 °C, with a final elongation step at 72 °C for 10 min. Amplified products were resolved electrophoretically in a 1.5% agarose gel. All reactions were performed twice. Following PCR amplification, some amplicons were cloned into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Subsequently, both amplicon strands were sequenced using the M13 forward and reverse primer. DNA sequences were aligned using the CLUSTALW algorithm to assess the potential intraspecific sequence variety. Sequences for each gene variant obtained is this study were deposited in GenBank under the accession numbers GQ268948–GQ268960.

Race determination/verification

Race 1 verification of a selection of *F. oxysporum* f. sp. *lycopersici* isolates was performed using the tomato lines GCR161, which contains the *I* gene and is resistant to race 1 isolates, and C32, which has general susceptibility to *F. oxysporum* f. sp. *lycopersici* (Kroon & Elgersma, 1993). Pathogenicity was tested using the root dip method (Wellman, 1939). Briefly, spores were collected from 5-day-old cultures in Czapek Dox broth (Difco) and used for root inoculation of 10-day-old plants at a spore density of 10^7 mL^{-1}. The seedlings were then potted individually and grown at 25 °C in a greenhouse. Three weeks after inoculation, disease was scored in two ways, including average plant weight above the cotyledons and phenotype scoring according to a disease index ranging from 0 (no symptoms) to 4 (heavily diseased or dead) (Rep *et al*., 2004).

Results and discussion

During infection, *F. oxysporum* f. sp. *lycopersici* secretes several unique, small proteins into the xylem sap that promote virulence of the fungus towards tomato (Rep *et al*., 2004; Houterman *et al*., 2007). Among them, all three predicted avirulence genes were found, including *AVR1* = *SIX4*, *AVR2* = *SIX3* and *AVR3* = *SIX1* (Rep *et al*., 2004; Houterman *et al*., 2008, 2009). The linkage between this group of genes and pathogenicity makes them potential host- and cultivar-specific pathogenicity markers. This hypothesis was tested by assessing the presence of the different *SIX* genes in a large, worldwide collection of fungal isolates (Table 1). All isolates listed in Table 1 were tested for the presence of the different *SIX* genes by PCR assays, using primers that anneal just outside the ORF. Most *SIX* genes were found to be present in the *forma specialis lycopersici*, but not in other *formae speciales* or nonpathogenic isolates (Table 1). While *SIX1*–*SIX5* are exclusively present in *F. oxysporum* f. sp. *lycopersici*, *SIX6* and *SIX7* amplicons were also generated from isolates of a few other *formae speciales*, including *lilii* (*SIX7*; 862 bp), *melonis* (*SIX6*; 793 bp) and *radicis-cucumerinum* (*SIX6*; 793 bp) (Table 1). Sequencing of these homologs revealed that they are identical within each *forma specialis*, but different between *formae speciales*, except for the *SIX6* homologs of the *formae speciales melonis* and *radicis-cucumerinum*, which are identical. This high conservation is in line with the high degree of conservation that was also observed for these and other *SIX* genes between isolates of *F. oxysporum* f. sp. *lycopersici* (Houterman *et al*., 2008, 2009; van der Does *et al*., 2008; this study). In comparison with *F. oxysporum* f. sp. *lycopersici*, 91% DNA sequence identity (ORF) and 84% amino acid identity was found for the *SIX7* homolog in *F. oxysporum* f. sp. *lilii*. For the *SIX6* homologs, 95% DNA sequence identity (ORF) and 90% amino acid identity was found between the *formae*

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speciales melonis/*radicis-cucumerinum* and *lycopersici*. Additional screening revealed that isolates of *F. oxysporum* f. sp. *vasinfectum*, pathogenic on cotton, also carry a homolog of *SIX6* (J. Ellis, pers. commun.). In accordance with Houterman *et al*. (2008), *SIX4* (*AVR1*) was generally found to be present in race 1 isolates only (Table 1), supporting its gene-for-gene relationship with the *I* resistance gene (Houterman *et al*., 2009). Remarkably, *SIX4* was absent in isolate CBS 645.78, which was previously classified as *F. oxysporum* f. sp. *lycopersici* race 1 (Table 1). On the other hand, a positive PCR was obtained for isolate CBS 646.78 and isolate MX395, which were previously determined as race 2 and race 3, respectively (Table 1). To check the racial identity of these isolates, a pathogenicity test on a tomato line carrying the *I* gene was performed, revealing that isolate CBS 645.78 was able to overcome the *I* gene and was therefore originally misidentified as race 1 isolate, while the last two isolates should be designated as race 1 isolates because they are avirulent on the *I* tomato line. In order to further assess the link between the unique presence of *SIX4* and race 1 designation (i.e. avirulence on an *I* tomato line), nine additional isolates of unknown race that were shown to have *SIX4* by PCR (i.e. CBS 164.85, CBS 165.85, CBS 758.68, DSM 62338, FOL 00/60309/1, FOL-295A, FRC-0-1113A, FRC-0-113N and NRRL 26034) were tested for their ability to cause disease on tomato seedlings containing the *I* resistance gene. All isolates turned out to be race 1, further demonstrating a perfect correlation between race 1 and the presence of *SIX4* as well as the ability to detect misidentifications with our PCR assay. The results of two of these pathogenicity assays are presented in Fig. 1. In order to differentiate race 2 from race 3 isolates, *SIX3* (*AVR2*) can be exploited. Indeed, race 2 strains were found to contain identical *SIX3* sequences (also identical to those of race 1 strains), which differ from race 3 strains by single point mutations (Houterman *et al*., 2009; Fig. 2). Three variants of this gene were found within race 3 isolates differing in a single nucleotide from race 1 and race 2 isolates (within the ORF: G121 > A, G134 > A and G137 > C; Fig. 2). Based on these nucleotide differences, three forward primers, SIX3-G121A-F2 (5′-ACGGGGTAACCCATATTGCA-3′), SIX3-G134A-F2 (5′-TTGCGTGTTTCCCGGCCA-3′) and SIX3-G137C-F1 (5′-GCGTGTTTCCCGGCCGCCC-3′), were developed and combined with the reverse primer SIX3-R2 (Table 2), enabling unambiguous PCR differentiation of race 2 and race 3 isolates when using stringent PCR conditions (i.e. using the PCR program described above, with the exception of an annealing temperature of 67 °C, an elongation time of 2 s and 30 instead of 35 cycles) (Table 3).

Rapid detection and reliable identification of potential plant pathogens is required for taking appropriate and

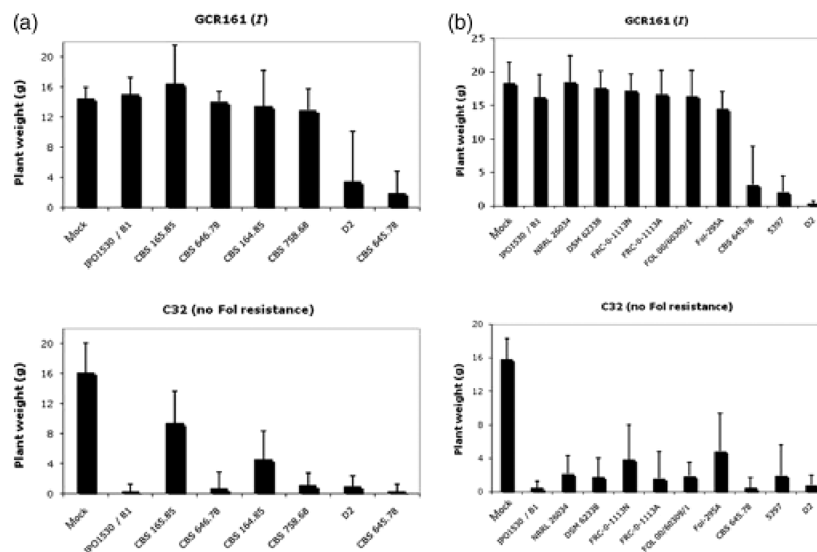

Fig. 1. Race determination of *Fusarium oxysporum* f. sp. *lycopersici* isolates by pathogenicity tests on tomato. To verify or determine the race of individual *F. oxysporum* f. sp. *lycopersici* isolates, tomato lines with single resistance genes were inoculated at the seedling stage and disease was scored 3 weeks after inoculation. Disease severity was measured by average plant weight above the cotyledons as compared with mock inoculation (mock). In the two experiments shown here (a and b), the plant line GCR161 was used to determine race 1 identity – this line contains only the *I* gene and is therefore resistant only to race 1. As controls, previously verified *F. oxysporum* f. sp. *lycopersici* isolates were included: IPO1530/B1 (race 1; alternative designation: Fol004), D2 (race 2; alternative designation: Fol007) and 5397 (race 3; alternative designation: Fol029). To assess the pathogenicity of each isolate, they were also inoculated on the generally susceptible tomato line C32 (i.e. without resistance genes against *F. oxysporum* f. sp. *lycopersici*). Apart from the race 2 and race 3 control strains, all the other strains tested in these two experiments were race 1, except for CBS 645.78, which is race 2 (avirulent on an *I-2* line, results not shown). Error bars indicate SD ($n = 20$).

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LSU-7      -GACGGGGTAACCCATATTGCGTGTTTCCCGGCCGCCGCACGTCTTCTACT-
FOL-MM10   -GACGGGGTAACCCATATTGCATGTTTCCCGGCCGCCGCACGTCTTCTACT-
14844      -GACGGGGTAACCCATATTGCGTGTTTCCCGGCCACCGCACGTCTTCTACT-
IPO3       -GACGGGGTAACCCATATTGCGTGTTTCCCGGCCGCCCCACGTCTTCTACT-
                                *            *  *
```

Fig. 2. Sequence alignment of part of the different versions of the *SIX3* gene found in *Fusarium oxysporum* f. sp. *lycopersici*. The LSU-7 sequence represents race 1 and race 2 sequences, whereas isolates FOL-MM10, 14844 and IPO3 represent race 3 strains. Variable nucleotides are underlined, marked in bold and with asterisks.

Table 3. Differentiation of *Fusarium oxysporum* f. sp. *lycopersici* race 3 from race 1 or race 2 isolates by PCR

		Specificity obtained with primers*		
Isolate	Race	SIX3-G121A-F2/SIX3-R2	SIX3-G134A-F2/SIX3-R2	SIX3-G137C-F1/SIX3-R2
IPO1530/B1	1	−	−	−
LSU-3	1	−	−	−
LSU-7	2	−	−	−
D2	2	−	−	−
FOL-MM10	3	+	−	−
FOL-MM25	3	+	−	−
14844	3	−	+	−
5397	3	−	+	−
IPO3	3	−	−	+
CA92/95	3	−	−	+

*+, amplicon detected; −, no amplicon detected. A positive signal for one of the three PCR reactions is expected for race 3 isolates. A fragment of 429, 414 or 412 bp is generated when using SIX3-G121A-F2/SIX3-R2, SIX3-G134A-F2/SIX3-R2 or SIX3-G137C-F1/SIX3-R2, respectively.

timely disease management measures. As DNA sequence variation in the commonly used housekeeping genes such as those for rRNA, β-tubulin or translation elongation factor-1α is not sufficient for the unambiguous identification of most *formae speciales* (O'Donnell *et al*., 1998; Lievens *et al*., 2007), other approaches are increasingly being used. These include, for example, methods based on the use of molecular markers identified by genotyping or amplification of transposon insertions (Lievens *et al*., 2008). Recently, another PCR method, based on polymorphisms in two genes encoding cell wall-degrading enzymes (*PG1* and *PGX4*), has been developed that distinguishes Japanese *F. oxysporum* f. sp. *lycopersici* strains (and its races) from Japanese *F. oxysporum* f. sp. *radicis-lycopersici* strains (Hirano & Arie, 2006). However, cross-reaction with other *formae speciales* was observed (Hirano & Arie, 2006), limiting the use of this method in practice. In contrast, our PCR screen showed a 100% success rate in discriminating *F. oxysporum* f. sp. *lycopersici* from other *formae speciales* (targeting *SIX1*, *SIX2*, *SIX3* and/or *SIX5*) and identification of the different races (targeting *SIX3* and *SIX4*). In addition, an experiment in which the presence of the pathogen was assessed in plant tissue showed that our PCR assays have the potential to detect and identify *F. oxysporum* f. sp. *lycopersici* in environmental samples, even before the plants have developed disease symptoms (data not shown). Consequently, our results pose potential benefits for tomato breeders and growers. These include, for example, accurate pathogen identification ensuring that effective control measures can be adopted or, conversely, unnecessary efforts can be avoided to control populations of *F. oxysporum* that are harmless (Lievens *et al*., 2008). In addition, our results can be used in breeding programs to evaluate or monitor disease resistance against specific pathogenic forms or races, for example by the development of quantitative real-time PCR assays enabling detection and quantification of pathogen biomass *in planta* (Brouwer *et al*., 2003). Apart from these practical implications, our results also shed new light on the possible origin of *formae speciales* of *F. oxysporum*. All *SIX* genes, except *SIX4*, are located on a single, relatively small chromosome (chromosome 14 in the sequenced *F. oxysporum* f. sp. *lycopersici* isolate 4287; http://www.broad.mit.edu/annotation/genome/fusarium_group/) (van der Does *et al*., unpublished data), and most of them are exclusively present in *F. oxysporum* f. sp. *lycopersici* (Table 1). Nevertheless, a few *SIX* genes (*SIX6* and *SIX7*) have close homologs in a few other *formae speciales* (Table 1). The ORFs of these homologs were found to be intact, indicating that these genes may have a function in pathogenicity and may be part of (a) different pathogenicity chromosome(s), with a gene content partly overlapping with chromosome 14. The *SIX* genes shared between different *formae speciales* may play a more general role in pathogenicity, while host specificity may be determined by a combination of unique genes. There are at least 122 transposons and 227 predicted genes, including the *SIX* genes, on chromosome 14 (M. Rep, unpublished data). The high transposon content of 'pathogenicity' chromosomes may explain why in several studies specific transposon-based markers could be linked to pathogenicity (Fernandez *et al*., 1998; Chiocchetti *et al*., 1999; Pasquali *et al*., 2004, 2007). Evolution of an ancestral pathogenicity chromosome within the *F. oxysporum* species complex and transfer between different VCGs (van der Does *et al*., 2008) may have given rise to a variety of host-specific pathogenicity chromosomes. Genome sequencing of isolates belonging to different *formae speciales* should reveal whether or not this is a likely scenario.

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