***Fusarium oxysporum* Evades I-3-Mediated Resistance Without Altering the Matching Avirulence Gene**

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I-3-Mediated resistance of tomato against *Fusarium* wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* depends on *Six1*, a protein that is secreted by the fungus during colonization of the xylem. Among natural isolates of *F. oxysporum* f. sp. *lycopersici* are several that are virulent on a tomato line carrying only the *I-3* resistance gene. However, evasion of *I-3*-mediated resistance by these isolates is not correlated with mutation of the *SIX1* gene. Moreover, the *SIX1* gene of an *I-3*-virulent isolate was shown to be fully functional in that i) the gene product is secreted in xylem sap, ii) deletion leads to a further increase in virulence on the *I-3* line as well as reduced virulence on susceptible lines, and iii) the gene confers full avirulence on the *I-3* line when transferred to another genetic background. Remarkably, all *I-3*-virulent isolates were of race 1, suggesting a link between the presence of *AVR1* and evasion of *I-3*-mediated resistance.

Additional keywords: gene-for-gene.

When analyzed genetically, many plant–pathogen interactions conform to the gene-for-gene concept. Simply stated, in a gene-for-gene interaction a single, dominant resistance (*R*) gene in the host and a corresponding avirulence (*Avr*) gene in the pathogen are required for disease resistance (Flor 1971; Van der Biezen and Jones 1998). Of course, many genes in the host are involved in disease resistance; also, in the pathogen, more than one gene may be required for expression of avirulence (Baker et al. 1997; Dangl and Jones 2001). Therefore, in more specific terms, a gene-for-gene interaction implies that the parents used in the genetic crosses—on the host side as well as the pathogen side—are polymorphic in just one gene that is required for resistance in the interaction under study, but not for disease resistance or pathogenicity in general. Therefore, it makes sense that most *R* genes that were cloned turned out to encode those elements of the innate immune system that specifically recognize either microbial molecules or plant components that are changed in a specific way by the pathogen; for instance, through proteolysis (Bonas and Lahaye 2002; Dangl and Jones 2001). In turn, the corresponding *Avr* genes usually encode secreted proteins that are either themselves specifically recognized by the matching *R* gene product or cause changes in the host that are recognized (Bonas and Van den Ackerveken, 1999; Laugé and De Wit 1998). Molecular arms races between

pathogens and their hosts are probably the cause of the relatively high variability in presence or sequence of *R* and *Avr* genes within populations or between related species or subspecific groups (De Wit and Joosten 1999; Ellis et al. 2000; Stahl and Bishop 2000).

In general, breaking of *R* gene-mediated resistance by pathogenic fungi has been associated with mutation of the matching *Avr* gene (Farman et al. 2002; Joosten et al. 1994; Luderer et al. 2002; Mandel et al. 1997; Orbach et al. 2000; Rohe et al. 1995; Van den Ackerveken et al. 1992). Here we present a case of a fungal pathogen that evades gene-for-gene resistance without mutation or loss of the avirulence factor.

The soilborne fungus *Fusarium oxysporum* causes Fusarium wilt disease in many plant species (Armstrong and Armstrong 1981). The existence of monogenic resistance (i.e., *R* genes) in many hosts, together with pathogenic races in host-specific forms of *F. oxysporum*, suggests that resistance to Fusarium wilt is, in many cases, based on specific recognition of molecular patterns. For *F. oxysporum* f. sp. *lycopersici*, this was further substantiated by subdivision of race 1 (avirulent on plants with the *R* gene *I* or *I-1*) into separate virulence groups based on virulence or avirulence on plants containing the *R* gene *I-2* (Mes et al. 1999). It also was from *F. oxysporum* f. sp. *lycopersici* that the first *Avr* gene of *F. oxysporum* was identified (Rep et al. 2004). This gene, *SIX1*, encodes a small, cysteine-rich protein that is secreted by *F. oxysporum* f. sp. *lycopersici* during colonization of the xylem of its host, tomato. Deletion of the *SIX1* gene breaks resistance mediated by the *R* gene *I-3*, qualifying *SIX1* as the *Avr* gene that matches *I-3*. *SIX1* is present in all tested isolates of forma specialis *lycopersici*, but not in several other isolates of *F. oxysporum* that either infect other plant species or have a different mode of infection (f. sp. *radicis-lycopersici*). This implies that *SIX1* may serve a role specifically in wilt disease of tomato.

Unexpectedly, some *F. oxysporum* f. sp. *lycopersici* isolates that are virulent on an *I-3* tomato line still contain an apparently intact *SIX1* gene (Rep et al. 2004). To investigate this further, we performed gene knock-out and complementation studies, which revealed that an isolate that is virulent on an *I-3* line still contains a fully functional *SIX1* allele. The implication is that evasion of *I-3*-mediated resistance by *F. oxysporum* f. sp. *lycopersici* isolates is due to suppression instead of changes in the *Avr* gene. We also demonstrate that *SIX1* is required for full virulence of *F. oxysporum* f. sp. *lycopersici* on susceptible tomato lines, which could explain why *SIX1* is present and presumably functional in all natural isolates of *F. oxysporum* f. sp. *lycopersici*.

RESULTS

Variation in Six1 protein sequence among natural isolates does not correlate with the degree of virulence on I-3 plants.

The cysteine-rich, in planta-secreted protein Six1 is required for avirulence of *F. oxysporum* f. sp. *lycopersici* on tomato line E779, which harbors the *I-3* resistance gene but no other resistance genes against *F. oxysporum* f. sp. *lycopersici* (Rep et al. 2004). In the common nomenclature for *F. oxysporum* f. sp. *lycopersici* races, the *I-3* gene confers resistance to race 3 in the *F. oxysporum* f. sp. *lycopersici*–tomato pathosystem (Table

Table 1. *Fusarium oxysporum* f. sp. *lycopersici* race nomenclature used in this study

Race	Tomato <i>R</i> gene		
	<i>I</i> or <i>I-1</i>	<i>I-2</i>	<i>I-3</i>
Race 1	Avirulent	Virulent or avirulent	Virulent or avirulent
Race 2	Virulent	Avirulent	Virulent or avirulent
Race 3	Virulent	Virulent	Avirulent

1). This system of nomenclature has a historical origin. Race 1 is, by definition, countered by the *I* or *I-1* gene. Race 2 is, by definition, virulent on *I* or *I-1* lines and is countered by the *I-2* gene. Race 3 is, by definition, virulent on the *I/I-1* and *I-2* lines and is countered by the *I-3* gene. However, race 1 is not defined in relation to the *I-2* gene and can be divided into separate virulence groups based on virulence on a line with only *I-2* (Mes et al. 1999). Likewise, race 1 and 2 are not defined in relation to the *I-3* gene. Therefore, we reasoned that among race 1 and 2 isolates, variation in virulence could exist on a line with *I-3* but no other *F. oxysporum* f. sp. *lycopersici* resistance genes. To see if this was the case, we tested virulence on E779 of 33 *F. oxysporum* f. sp. *lycopersici* isolates, representing three vegetative compatibility groups (VCG0030, VCG0031, and VCG0032) and all three races. These isolates all were highly virulent on the generally susceptible cv. MoneyMaker. Variation in virulence on E779 does indeed exist, but some care has to be taken to differentiate between “virulence” and “avirulence” (Fig. 1). In part, this is because avirulent interactions sometimes cause mild symptoms: a reduction in average plant weight (approximately 25% in this experiment, but usu-

Fig. 1. Variation in virulence among natural isolates of *Fusarium oxysporum* f. sp. *lycopersici* on the *I-3* line E779. The virulence of 33 isolates on 10-day-old seedlings of plant line E779 (*I-3*) was scored 3 weeks after infection by determining the average plant weight above the cotyledons (vertical axis, error bars indicate the 95% confidence interval) and average disease index (0 to 4, horizontal axis). To retain clarity, error bars are not shown for the average disease index; variation was in a range similar to that of plant weights. Ten plants were inoculated per isolate. The thick-lined open square on the vertical axis indicates the average plant weight of the mock-inoculated plants. Race 1 isolates are indicated with open squares, race 2 with gray squares, and race 3 with black squares. The *SIX1* allele (K or E) of isolates is indicated if known. Isolates discussed specifically in the text (Fol004, Fol007, Fol016, and Fol041) are indicated with numbers (Fol029 was not included in this experiment). The vertical dotted line marks an average disease index of 1.7, below which *F. oxysporum* f. sp. *lycopersici* isolates were considered to be avirulent. The horizontal dotted line marks an average plant weight of 50% of mock-inoculated plants, below which isolates were considered to be virulent. The five isolates in the upper right quadrant (average disease index above 1.7 but plant weight above 50% of control) were classified as intermediate.

ally less) and, in some plants, swelling of the hypocotyl (disease index 1) or mild vascular browning (disease index 2). In addition, disease development in virulent interactions is moderate (maximum average weight loss of 75%, no dead plants). Here, we consider isolates to be “avirulent” when the average disease index is less than 1.7, with no plants showing severe phenotypes. Isolates are considered “virulent” when the average weight loss is more than 50% and the average disease index is higher than 2. With these criteria, 21 of the 33 isolates tested were classified as avirulent (Fig. 1, upper left quadrant), 7 isolates were classified as virulent (Fig. 1, lower right quadrant) and 5 isolates (Fig. 1, top right quadrant) could not be assigned to either the avirulent or the virulent category and were classified as “intermediate”. One race 3 isolate, Fol029, was tested separately and was confirmed to be avirulent. Curiously, all virulent isolates as well as the five intermediate isolates are of race 1 (Fig. 1). Only 2 of the 14 race 1 isolates tested were classified as avirulent on E779 (Fol016 and Fol041), together with all 16 race 2 isolates tested (Table 2).

Having shown that natural variation in virulence on an *I-3* line exists, we tested whether this variation was related to polymorphisms in *SIX1*. The *SIX1* open reading frame was amplified

from all *F. oxysporum* f. sp. *lycopersici* isolates in our collection, which excludes loss of *SIX1* as a mechanism of overcoming *I-3* resistance by natural isolates (Rep et al. 2004). Therefore, we proceeded to see whether variation in the coding sequence could be responsible. *SIX1* was sequenced from 24 isolates that have different degrees of virulence on E779 and represent all three races. We found only the single nucleotide polymorphism reported earlier that leads to either a lysine or a glutamate at position 164 (Rep et al. 2004). Here, these two alleles are called the K allele and the E allele, respectively. Based on an earlier analysis of the phylogenetic relationship of many of the isolates used in this study (Mes et al. 1999), the K allele appears to be restricted to a subgroup of VCG0030, whereas the E allele is more widespread (Fig. 2). However, as shown in Figure 1 and summarized in Table 2, there is no correlation of either allele to virulence or avirulence on E779. These results exclude amino acid sequence variation as a cause of evasion of *I-3* resistance.

The *SIX1* gene from the *I-3*-virulent isolate Fol004 is functional.

Because neither loss nor mutation of the *Six1* protein can explain the observed virulence of some isolates on the *I-3* line,

Table 2. *Fusarium oxysporum* f. sp. *lycopersici* isolates used in this study

Isolate no.	Original designation	Source		Race	Virulence ^a	VCG ^b	<i>SIX1</i> allele ^c
		Donor	Origin				
Fol001	WCS801/E329	D. M. Elgersma	Netherlands	1	(v)	003-si	E
Fol002	WCS862/E241	N. A. M. van Stekelenburg	Netherlands	2	A	0030	K
Fol003	WCS861/E240	N. A. M. van Stekelenburg	Netherlands	1	nd	0030	K
Fol004	IPO1530/B1	IPO-DLO	Netherlands	1	V	0030	K
Fol005	C24/B2	IPO-DLO	Netherlands	2	nd	003-sc	E
Fol006	D1	L. Davidse	France	1	V	0030	K
Fol007	D2	L. Davidse	France	2	A	0030si	E
Fol008	E79	IPO-DLO	Netherlands	1	nd	003-sc	E
Fol009	FOL-R5-6/E172	R. J. Scheffer	Wisconsin, United States	1	V	0030si	E
Fol010	E175	D. M. Elgersma	Netherlands	1	V	0031	E
Fol011	E179	C. H. Beckman	United States	1	V	0030si	E
Fol012	E181	D. M. Elgersma	Netherlands	1	nd	003-si	E
Fol014	LSU-3	K. S. Elias	Louisiana, United States	1	(v)	0030	E
Fol015	BFOL-53	K. S. Elias (Black)	Louisiana, United States	2	A	0030	E
Fol016	BFOL-51	K. S. Elias (Black)	Louisiana, United States	1	A	0031	E
Fol017	OSU-451	K. S. Elias (Rowe)	Ohio, United States	2	A	0031	E
Fol018	LSU-7	K. S. Elias	Louisiana, United States	2	A	0032	E
Fol019	BFOL-70	K. S. Elias (Black)	Louisiana, United States	2	A	0032	E
Fol020	FRC 0-1078	T. Katan (Puhalla)	Florida, United States	2	A	0030	nd
Fol021	Fol1 (66044)	T. Katan	Israel	1	V	0030	nd
Fol022	Fol-650 B	T. Katan	Israel	1	V	0030	E
Fol023	Fol-1295T (66047)	T. Katan	Israel	2	A	0030	nd
Fol024	Fol-W841 D (76535)	T. Katan	Israel	2	A	nd	nd
Fol025	18947	D. J. McGrath	Australia	2	A	0030	nd
Fol026	14844 (M1943)	D. J. McGrath	Australia	3	nd (A)	0030	K
Fol028	548	J. P. Jones	Florida, United States	2	A	0030	nd
Fol029	5397	J. P. Jones	Florida, United States	3	A	0030	K
Fol032	48112	M. I. G. Roncero	Spain	1	(v)	003-si	nd
Fol033	281	M. I. G. Roncero	Spain	2	A	0030	nd
Fol034	4287	M. I. G. Roncero	Spain	2	A	0030	nd
Fol035	IPO3	IPO-DLO	Netherlands	3	A	0030	K
Fol036	...	J. W. Scott	Florida, United States	3	A	0030	K
Fol037a	MX395	Lawn	Mexico	3	nd (A)	0030	K
Fol038	CA92/95	Lawn	California, United States	3	A	0030	K
Fol039	EY-102	Suleman	Egypt	2	A	nd	nd
Fol041	MD-S2	Suleman	United States	1	A	nd	nd
Fol042	ATCC417	Suleman	United States	1	(v)	nd	nd
Fol043	ATCC605	Suleman	United States	2	A	nd	nd
Fol044	MD-L3	Suleman	United States	1	(v)	nd	nd
Fol045	T68	Van der Knaap	Italy	2	A	nd	nd

^a Virulence on E779: V = virulent; (v) = intermediate; A = avirulent; nd = not determined in this work; nd (A) = not determined in this work, but race 3 isolates should, by definition, be avirulent on E779.

^b VCG = vegetative compatibility group: 003-si = self-incompatible; 0030-si = self-compatible, but some compatibility reactions with other isolates within VCG0030; sc = self-compatible; nd = not determined. Data taken from Mes and associates (1999) and J. Mes (*unpublished data*).

^c Glutamate (E) or lysine (K) at position 164 (G or A at nucleotide 490); nd = not determined.

we assessed whether reduced *SIX1* gene expression could be responsible. As an example of an isolate that is virulent on E779, we focused on race 1 isolate Fol004, which has been extensively studied before (Teunissen et al. 2002, 2003) and contains the K allele (Fig. 1; Table 2). We compared this isolate to Fol007 (race 2, E allele) and Fol029 (race 3, K allele) that both are avirulent on E779. From sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels of xylem sap proteins from infected plants, we concluded that expression and secretion of *SIX1* is similar in the three isolates (Fig. 3). This is in line with the observation that the sequences upstream of *SIX1* in Fol004 and Fol007 are identical until at least 853 bp upstream of the ATG (Rep et al. 2004) (the Fol029 upstream sequence was not determined). Mass spectrometric analysis of the 12-kDa protein from Fol004-infected plants confirmed that it is the product of the *SIX1* gene. It was indistinguishable from the Six1 protein identified earlier in Fol007-infected plants (Rep et al. 2004) except for the predicted lysine at position 164 (results not shown). Apparently, the ability of Fol004 to cause disease on E779 is not due to altered expression of *SIX1*. To test this directly, the K allele from Fol004 was transformed to the *SIX1* knock-out strain Fol007 Δ six1 to check whether it could restore avirulence on E779. As a control, Fol007 Δ six1 also was transformed with the E allele from Fol007. E779 was inoculated with five independent transformants with the K allele and five transformants with the E allele. All were avirulent, except for one E allele transformant (E3), which showed only partial restoration of avirulence (Fig. 4). These results show unambiguously that the K allele from Fol004 is capable of inducing full *I*-3-mediated resistance in the Fol007 genomic context. Interestingly, deletion of *SIX1* in Fol004 leads to a further, significant increase in virulence on E779 (Fig. 5). Apparently, suppression of *I*-3-mediated resistance is not complete, at least in Fol004.

***SIX1* is required for full virulence in susceptible interactions.**

In the course of these experiments, we observed that, besides causing avirulence on *I*-3 plants, *SIX1* also has a role in virulence on susceptible plants. In bioassays with 10-day-old seedlings, this effect can be seen most clearly with the race 3 isolate Fol029 on C32 (no *F. oxysporum* f. sp. *lycopersici* resistance), GCR161 (only *I*), and 90E341F (only *I*-2) (Fig. 5). It also was observed in compatible interactions involving Fol004 (on C32

and 90E341F) and Fol007 (on GCR161) (Fig. 5). We reasoned that, with Fol004 and Fol007, these effects may be relatively small because infection of seedlings (with artificially damaged roots) obviates the need for Six1 as an important virulence factor. Indeed, a more pronounced effect was seen when 4- to 5-week-old plants without *F. oxysporum* f. sp. *lycopersici* resistance (C32) were inoculated (Fig. 6). Under these circumstances, *SIX1* knock-out strains either are strongly reduced in virulence (Fol004 Δ six1) or have lost pathogenicity altogether (Fol007 Δ six1 and Fol029 Δ six1). The strong reduction in virulence on susceptible plants resulting from deletion of *SIX1* in

Fig. 3. Six1 is present in xylem sap of tomato plants infected with various isolates of *Fusarium oxysporum* f. sp. *lycopersici*. Five-week-old susceptible (C32) tomato plants were infected with *Fusarium oxysporum* f. sp. *lycopersici* isolate Fol007 (7), Fol004 (4), or Fol029 (29) and xylem sap was collected 3 weeks later. Xylem sap proteins were separated on 15% Tris-Tricine gels and visualized with silver staining. Identities of the tomato pathogenesis-related (PR) proteins in Fol007-infected plants were determined in a previous study (Rep et al. 2002). Molecular weight markers are indicated on the left (in kDa).

Fig. 2. Phylogenetic distribution of the K and E alleles of *SIX1* among *Fusarium oxysporum* f. sp. *lycopersici* isolates. The phylogenetic relationship shown here between 19 *F. oxysporum* f. sp. *lycopersici* isolates and their assignment to vegetative compatibility groups (VCGs) was determined before (Mes et al. 1999). Race 1 isolates are indicated with open circles and race 2 with gray circles. The isolates with the K-allele among the isolates in this figure are Fol002, Fol003, Fol004, and Fol006. The others have the E allele (only Fol013 was not analyzed). Modified as previously published (Mes et al. 1999).

Fol004 is another indication that *SIX1* is functional in Fol004, despite triggering only partial resistance in E779.

Both the K and E alleles were able to restore virulence of Fol007 Δ six1 on older plants (Fig. 7). Remarkably, however, transformation with the K allele generally resulted in a higher level of virulence than transformation with the E allele. Apparently, the amino acid variation at position 164 is correlated with virulence on a susceptible tomato line rather than with avirulence on an *I-3* line.

DISCUSSION

We have shown conclusively that, even though deletion of *SIX1* leads to virulence on a plant line carrying only the *I-3* re-

sistance gene, evasion of *I-3* resistance by natural *F. oxysporum* f. sp. *lycopersici* isolates is not due to changes in the *SIX1* gene. From this, we conclude that induction of *I-3*-mediated resistance by Six1 can be suppressed by another fungal factor. Suppression of avirulence has been demonstrated before for plant-pathogenic bacteria, and can actually be mediated by avirulence factors (Abramovitch et al. 2003; Jackson et al. 1999; Tsiamis et al. 2000). Suppression (“inhibition”) of avirulence by unlinked loci also was demonstrated for the flax rust fungus in its interaction with flax (Jones 1988). No sexual cycle has been demonstrated for *F. oxysporum*; therefore, the existence of an avirulence suppressor locus cannot be investigated with classical genetics. If such a locus exists, it apparently is present only in race 1 isolates, at least in the collection that we

Fig. 4. Complementation of *Fusarium oxysporum* f. sp. *lycopersici* isolate Fol007- Δ six1 by the *SIX1* K allele from Fol004 restores avirulence. Ten-day-old seedlings of plant lines C32 (no *F. oxysporum* f. sp. *lycopersici* resistance, black bars) and E779 (*I-3* resistance gene, gray bars) were inoculated with Fol007 (007), the *SIX1*-deletion mutant Fol007 Δ six1 (Δ six1), or Fol007 Δ six1 transformed with the K allele of *SIX1* from Fol004 (K1-5) or the E allele of *SIX1* from Fol007 (E1-5) (five independent transformants each). Three weeks after infection, the average plant weight of 20 plants was determined (error bars: 95% confidence interval).

Fig. 5. Deletion of *SIX1* increases virulence on an *I-3* line of all *Fusarium oxysporum* f. sp. *lycopersici* races. Ten-day-old seedlings of plant lines C32 (no *Fusarium oxysporum* f. sp. *lycopersici* resistance, black bars), GCR161 (*I* gene against race 1, dark gray bars), 90E341F (*I-2* gene against race 2, gray bars), and E779 (*I-3* gene against race 3, white bars) were inoculated with Fol004 (race 1), Fol007 (race 2), Fol029 (race 3), or their respective *SIX1*-deletion mutants (Δ six1). Three weeks after infection, the average plant weight of 20 plants was determined (error bars: 95% confidence interval).

have investigated. This suggests that avirulence suppression might be linked to the putative *AVR1* gene that controls avirulence on plants carrying the *I* (or *I-1*) resistance gene. Indeed, in a parasexual cross of Fol004 (*AVR1*, *I-3*-virulent) and Fol029 (*avr1*, *I-3*-avirulent), the vast majority of the progeny inherited *AVR1* as well as virulence on the *I-3* line, which supports a link between *AVR1* and suppression of *I-3* resistance

(Teunissen et al. 2002). However, the skewed Fol004 parent-biased inheritance pattern of *AVR1* in this cross precludes firm conclusions about genetic linkage. We also note that the relationship between *AVR1* and evasion of *I-3* resistance is not strict. Although none of the 16 race 2 isolates tested were virulent on the *I-3* line, neither were 2 of the 14 race 1 isolates tested (Fig. 1; Table 2). Also, among the progeny of the

Fig. 6. Deletion of *SIX1* leads to reduced virulence on older plants. Five 4-week-old plants of plant line C32 (no *Fusarium oxysporum* f. sp. *lycopersici* resistance) were inoculated with Fol004 (race 1), Fol007 (race 2), Fol029 (race 3), or their respective *SIX1*-deletion mutants (Δ six1). Pictures were taken after 23 days.

Fol007

$\Delta six1$

K1

E3

K2

E4

K3

E5

Fig. 7. The *SIX1* K allele confers a higher degree of virulence to *Fusarium oxysporum* f. sp. *lycopersici* isolate Fol007- $\Delta six1$ than the E allele. Five 4-week-old plants of plant line C32 (no *F. oxysporum* f. sp. *lycopersici* resistance) were inoculated with Fol007, the *SIX1*-deletion mutant Fol007 $\Delta six1$ ($\Delta six1$), or Fol007 $\Delta six1$ transformed with the K allele (K) or E allele (E) of *SIX1* (five independent transformants each). Pictures were taken after 23 days. For each *SIX1*-allele, the results are shown of the three transformants that showed the highest degree of virulence.

parasexual cross described above, two recombinants were obtained that had the *AVR1* phenotype but were avirulent on the *I-3* line (Teunissen et al. 2002). This indicates that either suppression is not caused by *AVR1* but by a linked gene, or that suppression requires both *AVR1* and another gene.

In the *I-3*-virulent isolate Fol004, deletion of *SIX1* leads to a further increase in virulence on seedlings of E779. This implies that suppression of *I-3*-mediated resistance by natural isolates is not complete. The fact that all *F. oxysporum* f. sp. *lycopersici* isolates tested still contain *SIX1* is likely related to its requirement for full virulence in susceptible interactions, especially on older plants (Fig. 5). Therefore, although deletion of *SIX1* in a race 3 isolate can lead to a strain that is moderately virulent on seedlings with *I*, *I-2*, or *I-3* resistance and may hence be classified as race 4 (Fol029 Δ six1 in Fig. 5), the complete lack of pathogenicity of this strain on older plants probably disqualifies it as a serious threat to tomato in nature or agriculture.

A virulence function for an avirulence factor is not uncommon. As has been more extensively demonstrated for bacterial avirulence factors, proteins secreted in the host are likely to have a primary function in virulence, at least under some conditions (Van't Slot and Knogge 2002; White et al. 2000). For the pathogen, avirulence can be the sad consequence of recognition by the host of one of its virulence factors. In such cases, when complete loss of the *Avr* gene is no option, point mutations may be selected that destroy avirulence (i.e., *R* gene-mediated recognition) without loss of the virulence function. In *SIX1*, only a single nucleotide polymorphism was found among 24 *F. oxysporum* f. sp. *lycopersici* isolates, corresponding to position 164 in the central, 12-kDa part of Six1 that was found in xylem sap. There was no correlation to avirulence or virulence on the *I-3* line. Instead, the change of glutamate to lysine at this position, which apparently has occurred recently within a subgroup of VCG0030 (Fig. 2), can lead to enhanced virulence of *F. oxysporum* f. sp. *lycopersici* on a susceptible tomato cultivar (Fig. 7). Perhaps a point mutation in Six1 that could circumvent avirulence while retaining its virulence function simply has not yet occurred or spread within the population. Alternatively, such a mutation may not exist. This would be the case if *I-3* acts in accordance with the guard model, in which binding of the fungal protein to its virulence target in the plant is itself the trigger for resistance (Van der Hooft et al. 2002). Suppression by another factor is then the only option. Perhaps *AVR1*, or a gene linked to it, encodes a secreted protein (or is involved in production of a compound) that suppresses *I-3* resistance through interfering either directly with the *I-3* gene product or with downstream signal transduction processes.

MATERIALS AND METHODS

Plant material, fungal isolates, and infection assays.

The *F. oxysporum* isolates used for this study are presented in Table 2. The race nomenclature of *F. oxysporum* f. sp. *lycopersici* is as follows (Mes et al. 1999): race 1 is avirulent on *I/I-1* lines, race 2 is virulent on *I/I-1* but avirulent on *I-2* lines, and race 3 is virulent on *I/I-1* and *I-2* lines but avirulent on *I-3* lines (Table 1). The *SIX1* knock-out strain Fol007 Δ six1 was described before (Rep et al. 2004). *SIX1* knock-out strains Fol004 Δ six1 and Fol029 Δ six1 were constructed in a similar way, as described below.

The plant lines used in this study are Money Maker lines PV2002MM (Plant Breeding, Wageningen, The Netherlands) and C32 (Kroon and Elgersma 1993), which are susceptible to all races of *F. oxysporum* f. sp. *lycopersici*; GCR161, which contains the *I* gene and is resistant only to race 1 (Kroon and Elgersma 1993); 90E341F, which contains the *I-2* gene and is resistant to race 2 (Stall and Walter 1965); and E779, which

contains the *I-3* resistance gene and is resistant to race 3 (Scott and Jones 1989).

To test virulence of *F. oxysporum* f. sp. *lycopersici* isolates or mutants on tomato, the root dip method was used on either 10-day-old seedlings or 4- to 5-week-old plants (Wellman 1939). Briefly, spores were collected from 5-day-old cultures in potato-dextrose broth (BD, Sparks, MD, U.S.A.) and used for root-inoculation at a spore density of 10^7 /ml (seedlings) or 5×10^6 /ml (older plants). The plants then were potted individually. To score disease of seedlings, plant weight above the cotyledons was measured 3 weeks after inoculation and the extent of browning of vessels at the level of the cotyledons was scored. Disease index was scored on a scale of 0 to 4, where 0 = no symptoms; 1 = slightly swollen or bent hypocotyl; 2 = one or two brown vascular bundles in hypocotyl; 3 = at least two brown vascular bundles and growth distortion (strong bending of the stem and asymmetric development); and 4 = all vascular bundles are brown, plant either dead or very small and wilted.

Analysis of variance was done for all bioassays, but the resulting classifications were too complex to show in the figures. Instead, the 95% confidence intervals of the average plant weights are shown. As a general rule, when these intervals do not overlap between two inoculations in the same experiment, the difference between the averages is significant with $P < 0.01$. The large experiment shown in Figure 1 was done only once, but repeated testing of several isolates gave similar results.

Analysis of xylem sap proteins.

Collection of xylem sap and visualization of xylem sap proteins in SDS-PAGE gels was described before (Rep et al. 2002).

F. oxysporum f. sp. *lycopersici* transformation, genomic DNA extraction, PCR, and sequence analysis.

F. oxysporum f. sp. *lycopersici* was transformed with *Agrobacterium*-mediated transformation as described (Takken et al. 2004). For polymerase chain reaction (PCR) analysis, DNA was extracted from mycelium with a "quick" protocol in the following way. A small patch of mycelium was scraped from an agar plate and transferred to a 2-ml Eppendorf tube filled with 400 μ l of TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and approximately 300 μ l of glass beads. After addition of 300 μ l of phenol/chloroform (1:1), the suspension was vortexed for 2 min, followed by 5 min of centrifugation at top speed in an Eppendorf centrifuge. From the water phase, 0.2 μ l was used directly as template for PCR in microtiter plates with 20- μ l reaction mixes.

For deletion of *SIX1* in *F. oxysporum* f. sp. *lycopersici* isolates, pSIX1d::HYG was used (Rep et al. 2004). Transformants of *F. oxysporum* f. sp. *lycopersici* were tested for deletion of *SIX1* by PCR analysis, using primer P12-F19, which anneals upstream of the promoter sequence present in the construct used for transformation and primer, Trpc-F3, which anneals to the *TrpC* terminator behind the hygromycin resistance gene (Rep et al. 2004). The frequency of homologous recombination in *F. oxysporum* f. sp. *lycopersici* isolates varied from 3 to less than 1%.

For transformation of *SIX1* into *F. oxysporum* f. sp. *lycopersici*, pRD803-SIX1 (E allele) and pRD803-SIX1-K (K allele) were used, which contain the *SIX1* open reading frame from Fol007 or Fol004, respectively, flanked by 853 bp of upstream and 748 bp of downstream sequence. Selection was on zeomycin resistance. The plasmid with the K allele was constructed as described before for the E allele (Rep et al. 2004). Transformants of *F. oxysporum* f. sp. *lycopersici* were tested for the presence of the *SIX1* construct with complete flanks by PCR

analysis with primers M13-F (CAGCTATGACCATGATTACG; anneals to the left border of the T-DNA) and P12-R16 (GCCC CAAACCCAAGGATTG, anneals from +56 to +38 relative to the ATG). Only approximately 10% of zeomycin-resistant colonies contained the left border of the T-DNA, which—for unknown reasons—is much lower than with other, unrelated, T-DNA constructs (R. van Wijk, R. Duyvesteyn, and M. Rep, *unpublished data*).

The entire *SIX1* open reading frame was amplified from *F. oxysporum* f. sp. *lycopersici* isolates by PCR with primers P12-F2 (GTATCCTCCGGATTTTGAGC; anneals 30 to 11 bp upstream of the ATG) and P12-R1 (AATAGAGCCTGCAAA GCATG; anneals 106 to 87 bp downstream of the stop codon). The PCR fragments were sequenced directly on both strands with P12-F2 and P12-R1.

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