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The presence of a virulence locus discriminates *Fusarium oxysporum* isolates causing tomato wilt from other isolates

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Summary

Fusarium oxysporum **is an asexual fungus that inhabits soils throughout the world. As a species, *F. oxysporum* can infect a very broad range of plants and cause wilt or root rot disease. Single isolates of *F. oxysporum,* however, usually infect one or a few plant species only. They have therefore been grouped into *formae speciales* (f.sp.) based on host specificity. Isolates able to cause tomato wilt (f.sp. *lycopersici*) do not have a single common ancestor within the *F. oxysporum* species complex. Here we show that, despite their polyphyletic origin, isolates belonging to f.sp. *lycopersici* all contain an identical genomic region of at least 8 kb that is absent in other *formae speciales* and non-pathogenic isolates, and comprises the genes *SIX1*, *SIX2* and *SHH1*. In addition, *SIX3*, which lies elsewhere on the same chromosome, is also unique for f.sp. *lycopersici*. *SIX1* encodes a virulence factor towards tomato, and the Six1, Six2 and Six3 proteins are secreted in xylem during colonization of tomato plants. We speculate that these genes may be part of a larger, dispensable region of the genome that confers the ability to cause tomato wilt and has spread among clonal lines of *F. oxysporum* through horizontal gene transfer. Our findings also have practical implications for the detection and identification of f.sp. *lycopersici*.**

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Introduction

Soils harbour many diverse organisms, living together in complex networks of relations and interactions, some of which mutually beneficial, some parasitic. *Fusarium oxysporum* is a fungal species that is part of these networks and occurs in soils throughout the world. It is an asexual fungus that can cause wilt or root rot in many different plant species (Gordon and Martyn, 1997). As a species, *F. oxysporum* can infect a very broad range of hosts, among which are several economically important crops, like bulb flowers, cutting flowers, date palm, banana, tomato, melon and cucumber. Single isolates of *F. oxysporum,* however, usually infect only one or a few plant species. Isolates have therefore been grouped into *formae speciales* based on host specificity (Gordon and Martyn, 1997). An intriguing question is what enables a *F. oxysporum* isolate to infect a particular plant species, whereas another isolate will have a harmless or even a mutually beneficial relation to that same species (Recorbet *et al*., 2003).

The fact that *F. oxysporum* is an asexual fungus implies that between different isolates exchange of genetic information is limited. Genetic complementation between different isolates can only occur when the fusion partners belong to the same vegetative compatibility group (VCG). *Fusarium oxysporum* harbours many different VCGs, and molecular and genetic analyses have shown that isolates within a VCG are genetically similar and represent a clonal population (Corell, 1991; Koenig *et al*., 1997; Kistler *et al*., 1998; Katan and Katan, 1999). Several studies have shown that some *formae speciales* are composed of multiple VCGs and do not form a monophyletic group within the species. *Formae speciales cubense* (banana), *gladioli* (gladiolus) and *lycopersici* (tomato), for example, are polyphyletic (O'Donnell *et al*., 1998; Baayen *et al*., 2000). An important question is how isolates with a different origin have acquired pathogenicity towards the same host. Do all isolates of a *forma specialis* use the same virulence factors to attack their host?

In previous work we identified several genes from *F. oxysporum* f.sp. *lycopersici* (Fol) whose products are small proteins that are secreted into the host during infection (Rep *et al*., 2004; Houterman *et al*., 2007). Two of

Table 1. Presence of *SIX1* and *SIX2* in 287 isolates of *Fusarium oxysporum* and five isolates from other *Fusarium* species, as assessed by PCR.

	Number of isolates		
Isolate	*SIX1* and *SIX2* present[a]	None present	Total
Fusarium oxysporum			
f.sp. *lycopersici*	77	5	82
f.sp. *radicis-lycopersici*	1	74	75
14 *other f.*spp.	0	130	130
Fusarium graminearum	0	1	1
Fusarium solani	0	4	4
Total			292

a. All isolates that contain *SIX1* also contain *SIX2* and vice versa.

these genes, *SIX1* and *SIX2* (secreted in xylem 1 and 2), are located within 8 kb of each other. *SIX1* was shown to be required for full virulence (Rep *et al*., 2005a). In the present study, we tested a worldwide collection of *F. oxysporum* isolates, representing many different *formae speciales* and VCGs for the presence of *SIX1* and *SIX2*. We show that both genes are unique to f.sp. *lycopersici*, and are present in the entire polyphyletic group of f.sp. *lycopersici* isolates. The same distribution was observed for *SIX3*, another gene for a small, in xylem-secreted protein present on the same chromosome. Not only do these findings shed new light on the possible origin of host specificity, they can also be used to develop new methods for specific detection of Fol isolates.

Results

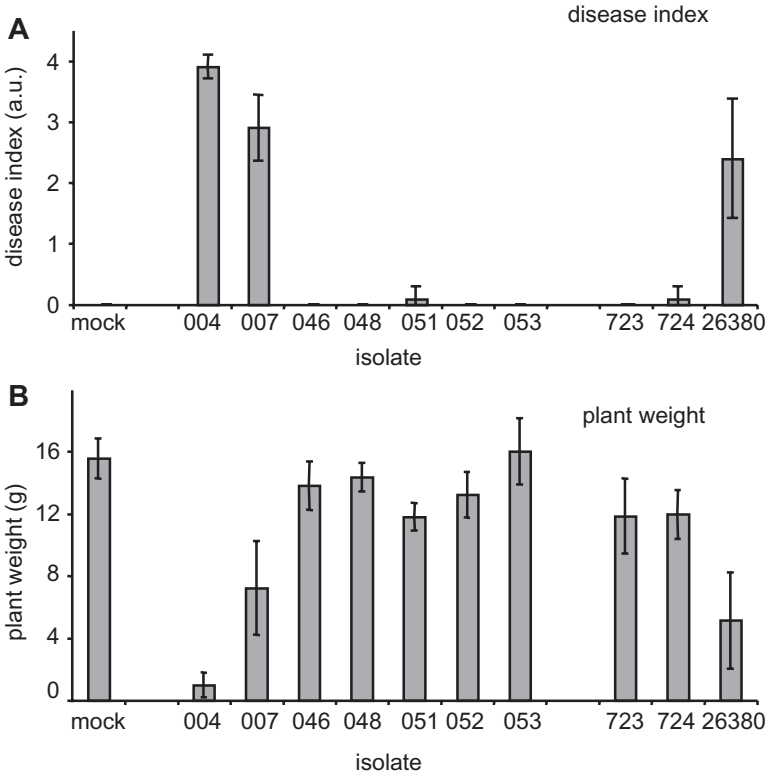
To test how the *SIX1* gene is distributed among different *formae speciales* of *F. oxysporum*, a large collection of isolates was screened. The collection consists of 287 *F. oxysporum* isolates, one *F. graminearum* isolate and four *F. solani* isolates (Table S1). Among the *F. oxysporum* isolates 82 were classified as f.sp. *lycopersici* (Fol) and 75 as f.sp. *radicis-lycopersici* (Forl). These two forms are both pathogenic towards tomato, but while Fol causes wilt, Forl causes foot and root rot. The presence of *SIX1* was assessed by polymerase chain reaction (PCR), using primers that anneal just outside the ORF. In total, 78 isolates yielded a PCR product of 992 bp, of which 77 were classified as Fol and one as Forl. Among the isolates that did not yield a PCR product five were classified as Fol (Table 1).

In the Fol strains in which we originally identified *SIX1* (Fol004 and Fol007), a second gene for a small, secreted protein was found, called *SIX2*. The *SIX2* gene is located within 8 kb of *SIX1* (Houterman *et al*., 2007). The two genes do not share any sequence homology. The collection of *Fusarium* isolates was also screened for the presence of *SIX2* by PCR, using primers outside the ORF. The same isolates that yielded a *SIX1* product also yielded a *SIX2* amplicon, with a length of 749 bp. All other isolates were negative for both *SIX1* and *SIX2*.

The presence of *SIX1* and *SIX2* was almost exclusive for f.sp. *lycopersici*, however, six exceptions were found. These six isolates were tested for their ability to cause tomato wilt (Fig. 1). It appeared that all five isolates that were classified as Fol but that do not have *SIX1* and *SIX2,* are in fact not able to cause tomato wilt. Therefore, by definition, these should not be considered Fol isolates. In contrast, the only Forl isolate that contains *SIX1* and *SIX2* was clearly able to cause wilt in tomato, and should therefore be designated as a *lycopersici* isolate. Taking this bioassay into account, there is 100% correlation between the ability to cause tomato wilt and the presence of *SIX1* and *SIX2*. Moreover, the PCR method we used is robust enough to detect previous misclassifications.

Previously, O'Donnell and colleagues (1998) published data that show that Fol is not a monophyletic group. This raises the question whether *SIX1* and *SIX2* are really absent in non-*lycopersici* isolates most closely related to Fol isolates, even though they were not detected by PCR. To test that possibility, Southern blots were performed with a representative subset of isolates. The selected Fol isolates encompassed four different VCGs (0030, 0031, 0032 and 0033), several isolates that were used in previous studies (O'Donnell *et al*., 1998; Baayen *et al*., 2000) and, finally, the five isolates that were originally classified as *lycopersici*, but did not cause tomato wilt disease and were negative for *SIX1* and *SIX2* in the PCR assay. Next to these, isolates from other *formae speciales* were selected based on their position in the phylogenetic trees from O'Donnell and colleagues (1998) and Baayen and colleagues (2000), including the isolate that was originally classified as Forl, but can cause tomato wilt disease as demonstrated in this paper. Details on the isolates used can be found in Table 2.

All isolates that yielded a *SIX1* PCR product [and caused tomato wilt (Fig. 2A)] show two hybridizing bands on a Southern blot when probed with the *SIX1* probe (Fig. 2B, upper panel). The lower hybridizing band is the



C

-	Fol	Fol	Fol	Fol	Fol	Fol	Fol	Forl	Forl	Forl
-	*SIX1*	*SIX1*	-	-	-	-	-	-	-	*SIX1*
-	*SIX2*	*SIX2*	-	-	-	-	-	-	-	*SIX2*

Fig. 1. The ability to cause tomato wilt correlates with the presence of *SIX1* and *SIX2*. Tomato seedlings were inoculated with different Fol or Forl isolates: 'mock', mock inoculation (water only), '004' and '007', known Fol isolates (controls), '046', '048', '051', '052' and '053', isolates previously classified as Fol, without *SIX1* and *SIX2,* '723' and '724', known Forl isolates (controls) and '26380', the isolate with *SIX1* and *SIX2* previously classified as Forl. Disease was measured 3 weeks after inoculation. The bars indicate the average of 20 plants, with error bars indicating the 95% confidence interval.
A. Disease severity, in arbitrary units (0, no symptoms; 1, slightly swollen and/or bent hypocotyl; 2, one or two brown vascular bundles in hypocotyl; 3, at least two brown vascular bundles and growth distortion; 4, all vascular bundles are brown, plant either dead or very small and wilted).
B. Plant weight, in grams.
C. Overview of the original classification (Fol or Forl), and presence of *SIX1* and *SIX2* in the isolates tested.

SIX1 gene, the other band contains a gene with high similarity to *SIX1*, called *SIX1-H*. *SIX1-H* is a known homologue of *SIX1*, of which the ORF has been truncated by the insertion of a transposon (Rep *et al*., 2005b). When probed with *SIX2*, the same isolates also show two hybridizing bands (Fig. 2B middle panel). One has the predicted size for the *SIX2* locus, the other is of unknown origin. The latter locus will be referred to as *SIX2-H*, even though the corresponding sequence is unknown. The isolates that did not yield *SIX1* and *SIX2* PCR products also do not show the double sets of hybridizing bands. However, sequences more distantly related to *SIX1* and/or *SIX2* appear to be present in some of those isolates, as evidenced by the weakly hybridizing bands of different sizes. In these isolates the presence of *SIX1*-related sequences is not coupled to *SIX2*-related sequences.

All *SIX1* and *SIX2* genes present in the isolates used for the Southern blot were amplified by PCR (Fig. 2C) and sequenced. All *SIX2* sequences were identical and for *SIX1* only one single nucleotide polymorphism was found, resulting in either a glutamine or a lysine at position 164 of the protein. The lysine ('K variant') was found in some isolates of VCG 0030, consistent with earlier findings (Rep *et al*., 2005a) but also in VCG 0033.

As the presence of *SIX1* and *SIX2* is always coupled, and as they are located close to each other (Fig. 3A), it might be that the entire region is unique to Fol. To test this, the presence of the gene that lies between *SIX1* and *SIX2* was assessed by PCR for the isolates used for the Southern blot (Fig. 2C). The presence of this gene, called *SHH1 (*salicylate hydroxylase homologue 1), is indeed coupled to the presence of *SIX1* and *SIX2*, as is the presence of the *SIX1* homologue (*SIX1-H*), mentioned above (Fig. 2C). From the isolates containing *SIX1*, *SIX2* and *SHH1* part of the non-coding region between *SIX1* and *SHH1* was sequenced (908 bp) and not a single nucleotide polymorphism was found (Fig. 3A).

The unique presence of an almost identical genomic region of at least 8 kb in a polyphyletic group of isolates is surprising. To confirm the polyphyletic nature of the isolates containing the *SIX1/2*-cluster, a phylogenetic tree was assembled using *EF1-*α and *mtSSU* sequences generated by (O'Donnell *et al*., 1998), (Baayen *et al*., 2000) and newly generated sequences of the Fol isolates rep-

Table 2. Isolates selected for detailed analysis.

Isolate no.	Forma specialis	Host	NRRL	Original name	Origin	VCG	SIX1 allele
Fol 004	*lycopersici*	Tomato		IPO 1530/B1	Netherlands	VCG 0030	K
Fol 007	*lycopersici*	Tomato		D2	France	VCG 0030	E
Fol 002	*lycopersici*	Tomato		WCS862/E241	Netherlands	VCG 0030	K
Fol 010	*lycopersici*	Tomato		E175	Netherlands	VCG 0031	E
Fol 018	*lycopersici*	Tomato		LSU-7	Louisiana, USA	VCG 0032	E
Fol MM-10	*lycopersici*	Tomato		FOL-MM10	Arkansas, USA	VCG 0033	K
Fol 26203	*lycopersici*	Tomato	26203				E
Fol 26383	*lycopersici*	Tomato	26383				K
Fol 26200	*lycopersici*	Tomato	26200				E
Fol 046[a]	Unknown	Unknown		MUCL 14159			–
Fol 048[a]	Unknown	Unknown		CBS 249.52			–
Fol 051[a]	Unknown	Unknown		DSM 62060	USA		–
Fol 052[a]	Unknown	Unknown		NRRL 22544	–		
Fol 053[a]	Unknown	Unknown		E184	Bulgaria		–
Forl 26381	*radicis-lycopersici*	Tomato	26381				–
Forl 26033	*radicis-lycopersici*	Tomato	26033				–
Forl 26380[b]	*lycopersici*	Tomato	26380				K
Fom 26406	*melonis*	Melon	26406		USA	VCG 0136	–
Fot 26954	*tulipae*	Tulip	26954	LBO Tu10	the Netherlands	VCG 0230	–
Foa 28379	*asperagi*	Asperagus	28379	FGSC 6621	USA	VCG 1008	–
Foa 28362	*asperagi*	Asperagus	28362	FGSC 6609	USA	VCG 1002	–
Foli 28395	*lilli*	Lily	28395	CPRO Fol028	Italy	VCG 0190	–
Fod-128356	*dianthi*	Carnation	28356	Garibaldi F639	Italy	VCG 0022	–
Fod-228401	*dianthi*	Carnation	28401	IPO WCS816	the Netherlands	VCG 0021	–
Foc 25609	*cubense*	Banana	25609	Kistler MW2	Malawi	VCG 01214	–
Foc 25603	*cubense*	Banana	25603	Kistler A2	Australia	VCG 0120	–
Forl 723	*radicis-lycopersici*	Tomato		C63F	Israel		–
Forl 724	*radicis-lycopersici*	Tomato		C142	Israel		–
Fo-47	Non-pathogenic	–		Fo-47	France		–
F. 22903	*Fusarium* sp.	*P. mensiesii*	22903				n.d.

a. Originally identified as f.sp. *lycopersici*.
b. Originally identified as f.sp. *radicis-lycopersici*.
n.d.: not determined.

resenting the different VCGs (Fig. 3B). The different VCGs appear in different clades, as expected. VCG 0030 and 0032 cluster together, in accordance with previous observations (Elias *et al*., 1993; Mes *et al*., 1999). Unexpectedly, the E and the K alleles of *SIX1* show a polyphyletic distribution; both appear in different clades and VCGs. The E-allele is present in VCG 0032 and VCG 0031 and, although not visible in this figure, also in VCG 0030 (Rep *et al*., 2005a). The K-allele is present in VCG 0033 and also in VCG 0030. So it appears that in two clades (VCG 0031 and VCG 0033) only one of both alleles is present, whereas in the clade that includes VCG 0032 and VCG 0030 both alleles are represented.

Because all Fol isolates carry the same genomic region, we wondered whether the presence of this region is the reason that these isolates are able to cause tomato wilt. Because *SIX1* is the only known virulence factor of this region, *SIX1* was transformed to several different *formae speciales* using a construct that can complement a *SIX1* null mutation, and four transformants of each strain were tested for their ability to cause tomato wilt. The recipient strains were *Fo* f.sp. *melonis* (26406, Fig. 3B), *Fo* f.sp. *radicis-lycopersici* (Forl 723) and a non-pathogenic strain (Fo-47). None of the transformants was able to cause tomato wilt disease, indicating that more genes are required to cause tomato wilt (Fig. S1). These genes remain to be identified, but might include *SIX2* and *SHH1*, and perhaps also *SIX3*, another small, secreted protein from Fol that was identified recently (Houterman *et al*., 2007). With the release of the *F. oxysporum* f.sp. *lycopersici* genome sequence (http://www.broad.mit.edu/annotation/genome/fusarium_group.1/MultiHome.html), we discovered that *SIX3* is located on the same supercontig (supercontig 36) as *SIX1* and *SIX2*, approximately 103 kb away from *SIX2*. To test if *SIX3* may be part of the genomic region unique for f.sp. *lycopersici* isolates we tested our collection of 292 isolates with primers just outside the *SIX3* ORF. This experiment revealed that, just like *SIX1* and *SIX2*, *SIX3* is present in all *F. oxysporum* isolates that are able to cause tomato wilt, but not in any of the other isolates. In Fig. 2 the PCR results for *SIX3* on a representative set of isolates are shown. In conclusion, *SIX1*, *SIX2* and *SHH1* are part of a genomic region that is only present in isolates that cause tomato wilt, is at least 8 kb but may stretch all the way to *SIX3* and perhaps even further.

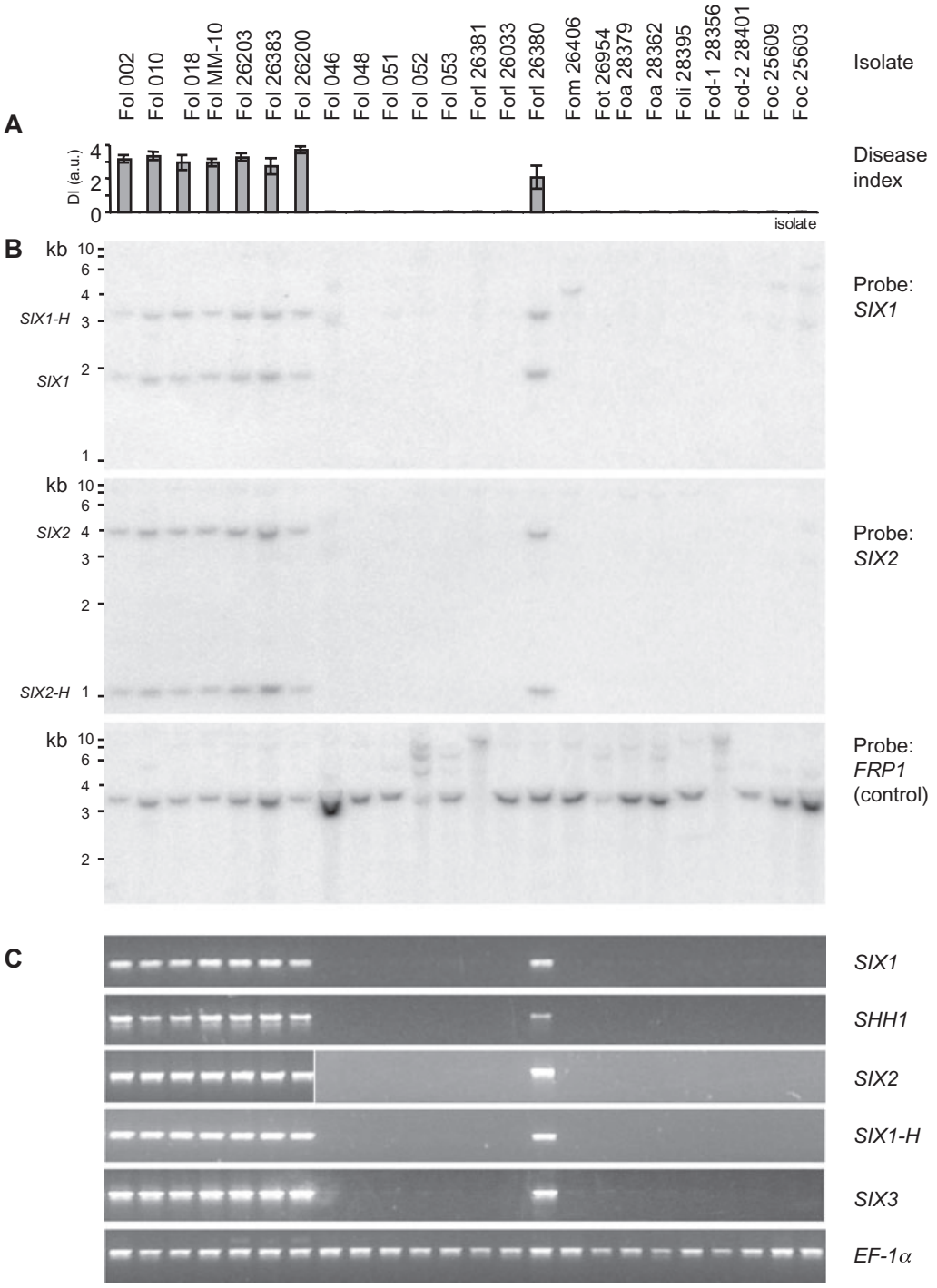


Fig. 2. The presence of *SIX1* and *SIX2* and related sequences in a selection of *formae speciales* of *F. oxysporum*.
A. The ability of the different isolates to cause tomato wilt. Tomato seedlings were inoculated with different isolates and disease was measured three weeks after inoculation. The bars indicate the average disease index (DI) of 10 plants, with error bars indicating the 95% confidence interval. DI was measured in arbitrary units (0, no symptoms; 1, slightly swollen and/or bent hypocotyl; 2, one or two brown vascular bundles in hypocotyl; 3, at least two brown vascular bundles and growth distortion; 4, all vascular bundles are brown, plant either dead or very small and wilted).
B. Southern blots with genomic DNA of the same isolates, digested with NcoI and XhoI and probed with *SIX1*, *SIX2* or *FRP1* (control).
C. PCR on genomic DNA of the same isolates, using primers amplifying *SIX1*, *SIX2*, *SHH1*, *SIX1-H, SIX3* or part of elongation factor 1α (*EF-1*α, control).
The strains in this figure are listed in Table 2 with more detailed information. Fol, f.sp. *lycopersici*, Forl, f.sp. *radicis-lycopersici*, Fom, f.sp. *melonis*, Fot, f.sp. *tulipae*, Foa, f.sp. *asparagi*, Foli, f.sp. *lilii*, Fod, f.sp. *dianthi*, Foc, f.sp. *cubense*.

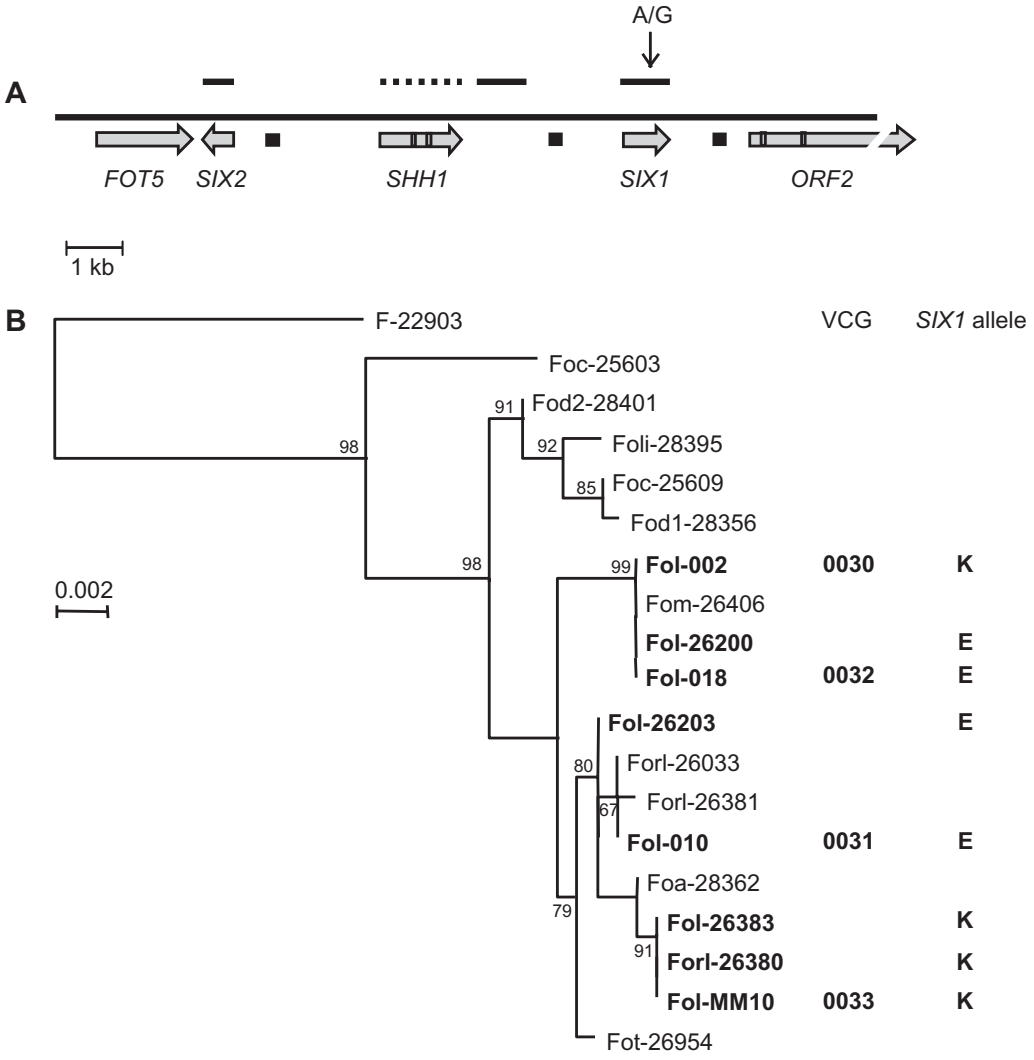


Fig. 3. A. The genomic locus of *SIX1*. Below the long black line are indicated: genes (block arrows, small internal blocks show the position of introns), gene names and miniature impala transposable elements (black blocks). Above the line are indicated the regions that were sequenced and/or amplified in all Fol isolates and Forl 26380. A dashed line indicates that the fragment was only amplified, a normal line that the fragment was also sequenced. The only polymorphism is indicated. *FOT5* and *ORF2* are predicted to be transposable elements.
B. Neighbour-joining tree of the *EF-1α* and *mtSSU* sequences of all isolates used for detailed study in this paper. The scale bar represents 0.002 changes per basepair. Bootstrap values (1000 replications) are indicated at tree nodes. All isolates causing tomato wilt are shown in bold letters. For all of these the *SIX1* allele (E or K) is indicated, and for some (when known) the VCG they belong to.

Discussion

Isolates of *F. oxysporum* that cause tomato wilt disease do not form a monophyletic group; they appear scattered over phylogenetic trees of the *F. oxysporum* species complex (O'Donnell *et al.*, 1998; Baayen *et al.*, 2000) (this study). One interpretation of this is that the ability to cause wilt in tomato has evolved several times independently. However, based on the results presented here, we believe that: (i) the ability to cause tomato wilt disease evolved only once; (ii) the genes described in this paper are part of a genomic region that is responsible for the ability to cause tomato wilt; and (iii) after this region emerged in one isolate, it spread to other clonal lines, possibly through horizontal gene transfer (HGT). Three main observations support this scenario. The first is that there is a 100% correlation between the presence of the investigated genes and the ability to cause tomato wilt disease (including complete lack of these genes in all other isolates). This supports the idea that these genes, by themselves or as part of a larger group, are responsible for this ability. In fact, *SIX1* has already been shown to positively contribute to virulence, and the Six1 as well as the Six2 and Six3 proteins are secreted into the xylem sap of infected tomato plants, suggesting a function in colonization for Six2 and Six3 as well. The exact set of

genes or the genomic region required for host specific virulence remains to be determined. *SIX1* by itself is not sufficient to introduce the ability to infect tomato plants in non-*lycopersici* isolates, but a bigger region might be. Interesting, in this respect, is a spontaneous mutant (F1-27) (Rep *et al*., 2004) that has lost an estimated 20–50 kb, including *SIX1*, *SIX2*, *SIX2-H* and *SHH1* but not *SIX3* (data not shown), and is still virulent on tomato plants, although reduced, similar to a *SIX1*-deletion mutant (Rep *et al*., 2005a). This implies that genes other than *SIX1*, *SIX2*, *SIX2-H* and *SHH1* contribute to the development of vascular wilt disease in tomato. We are investigating whether *SIX3* may be one of these genes.

The second observation supporting HGT is that the group of genes we investigated appears to be identical in each Fol isolate tested, with the exception of a single nucleotide polymorphism in the *SIX1* gene. Even in over 900 bp of non-coding region no polymorphism was found. When compared with the *EF-1α* sequences, which show 11 polymorphic sites in 389 bp of intron sequence between the same Fol isolates, this is an impressive lack of divergence. In addition, no differences in restriction fragments on Southern blot were observed. This strongly suggests that this region has been transferred between clonal lines.

The third observation supporting HGT is that most, or perhaps all, genes investigated are located on the same chromosome. *SIX1*, *SIX2* and *SHH1* are located within 8 kb of each other. *SIX1-H* and *SIX3* are located further away, but on the same chromosome (Rep *et al*., 2005b) (http://www.broad.mit.edu/annotation/genome/fusarium_group.1/MultiHome.html). Although *SIX2-H* is not present in the current assembly of the Fol genome sequence, it is absent in the spontaneous chromosomal deletion mutant F1-27 together with *SIX1* and *SIX2*, suggesting close proximity as well.

Fusarium oxysporum is not the first plant pathogenic fungus for which HGT of virulence genes is suggested. *Pyrenophora tritici-repentis,* which causes tan spot disease on wheat, produces the host selective toxin ToxA, which is sufficient to make a non-pathogenic isolate pathogenic (Ciuffetti *et al*., 1997). A related wheat-infecting fungus, *Stagonospora nodorum*, was recently found to harbour a close homologue of the *TOXA* gene (Friesen *et al*., 2006). Other species, more closely related to *P. tritici-repentis* but with a different host range, do not have *TOXA* sequences, and the two *TOXA* sequences of *P. triciti-repentis* and *S. nodorum* are more similar than can be expected based on the rest of their respective genomes (Friesen *et al*., 2006). The shared region encompasses 11 kb, includes *TOXA* and a transposase gene and is bordered by repetitive sequences in *S. nodorum*. Together, these observations strongly suggest HGT. In *Nectria haematococca,* VanEtten and colleagues found an entire chromosome of 1.6 Mb which is conditionally dispensable (CD) and harbours genes responsible for high virulence towards pea (Han *et al*., 2001). When the CD chromosome is transferred to an isolate from the same species that does not contain this chromosome, this isolate becomes highly virulent. Similar to pathogenicity islands in bacteria (Hacker and Kaper, 2000), it has a high number of transposable elements and a different GC content compared with the rest of the genome (Liu *et al*., 2003; Temporini and VanEtten, 2004). In the case of Fol examined here, the genes required for pathogenicity towards tomato involve more than just *SIX1*, but the involvement of an entire (CD) chromosome remains speculative. The chromosome with *SIX1* does show some features typical of a CD chromosome; it is the second smallest chromosome (~2 Mb) and is highly enriched in transposable elements (Rep *et al*., 2004; Rep *et al*., 2005b; M. Rep, unpubl. obs.).

The possibility of HGT raises several questions, one of which concerns the mechanism involved. Transmission of entire chromosomes or parts of chromosomes requires donor and acceptor hyphae to fuse and genetic material to be mixed. In the case examined here, donor and acceptor hyphae would not belong to the same VCG, which would normally restrict exchange of genetic material. However, in *Colletotrichium gloeosporoides*, transfer of a supernumerary chromosome (2 Mb) under laboratory conditions has been reported between vegetatively incompatible isolates, showing that genetic material can in some cases be exchanged across VCGs (He *et al*., 1998). If true, the HGT events proposed here for Fol seem to occur on a regular basis. Based on the distribution of Fol isolates in the phylogenetic tree (Fig. 3B), there must have been at least two recent HGT events. As VCG 0030 contains both the E and K allele of *SIX1*, the simplest scenario would be one transfer of the E-allele from VCG 0030/32 to VCG 0031 and another transfer of the K allele from VCG 0030 to VCG 0033.

Apart from these speculations, our findings have practical implications. Discrimination between pathogenic and non-pathogenic *formae speciales* towards a specific crop could prevent unnecessary efforts to suppress harmless fungal populations. In fact, non-pathogenic isolates may even contribute in a positive way plant growth, for instance via biocontrol (Mandeel and Baker, 1991; Diedhiou *et al*., 2003; Forsyth *et al*., 2006). The need to identify *formae speciales* with molecular methods, without recourse to a bio-assay, was already expressed several years ago (Recorbet *et al*., 2003). Just recently, a method has been published which distinguishes Japanese Fol isolates from Forl isolates, based on polymorphisms in two polygalacturonase genes (*PG1* and *PGX4*). However, cross-reaction with other *formae speciales* with this method was observed (Hirano and Arie, 2006). The PCR

screen with *SIX1* or *SIX2* primers we used showed a 100% success rate in identifying f.sp. *lycopersici* isolates in a large, worldwide collection. This shows that identification of *formae speciales* based on host-specific virulence genes could be very robust.

Experimental procedures

Bioassay on tomato

The tomato cultivar used in this study was Moneymaker C32, which is susceptible to all races of Fol (Kroon and Elgersma, 1993). To test pathogenicity of the different *F. oxysporum* isolates on tomato, the root dip method was used (Wellman, 1939). Briefly, spores were collected from 5-day-old cultures in Czapek Dox broth (CDB; Difco) and used for root inoculation of 10-day-old tomato plants at a spore density of 10^7 spores per millilitre. The seedlings were then potted individually and grown at 25°C in the greenhouse. Three weeks after inoculation, plant weight above the cotyledons was measured, and the extent of browning of vessels in the remaining part of the stem was scored. Disease index was scored on a scale of 0–4: 0, no symptoms; 1, slightly swollen and/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); 4, all vascular bundles are brown, plant either dead or very small and wilted.

Fusarium *isolates*

A collection of 292 *F. oxysporum* isolates, obtained from several culture collections or kindly provided by colleagues, was used. One hundred seventy-seven *F. oxysporum* strains isolated from tomato were used, encompassing 20 non-pathogenic strains, 82 isolates belonging to *F. oxysporum* f. sp. *lycopersici* and 75 isolates belonging to *F. oxysporum* f. sp. *radicis-lycopersici*, representing most known VCGs of these formae speciales (Table S1). In addition, the fungal strain collection contained 130 *F. oxysporum* isolates of 14 other formae speciales and five isolates of two other *Fusarium* species (Table S1). Many of these isolates have been characterized with respect to pathogenicity, vegetative compatibility and genetic diversity in previous studies (Baayen *et al*., 2000; Balmas *et al*., 2005) (Katan *et al*., 1991; Marlatt *et al*., 1996; O'Donnell *et al*., 1998; Katan and Katan, 1999; Vakalounakis and Fragkiadakis, 1999; Punja and Parker, 2000; Skovgaard *et al*., 2001; Skovgaard *et al*., 2002; Vakalounakis *et al*., 2004; Cafri *et al*., 2005). Isolates were grown in the dark at 22°C on potato dextrose agar containing 100 p.p.m. streptomycin sulfate.

PCR screen for determining the presence of SIX1*,* SIX2 *and* SIX3

All isolates listed in the Table S1 were tested for the presence of *SIX1*, *SIX2* and *SIX3* by a PCR screen using primer pairs located just outside the respective ORFs. Genomic DNA was extracted using the phenol-chloroform extraction method as described earlier (Lievens *et al*., 2003). As a check for DNA quality, all DNA samples were successfully subjected to PCR analysis using the universal primers ITS5 and ITS4, targeting the ribosomal RNA gene (Table 3).

In order to determine the presence of *SIX1*, primers P12-F2B and P12-R1 were used, for the determination of the presence of *SIX2*, primers SIX2-F2 and SIX2-R2 and for determination of the presence of *SIX3* primers SIX3-F1 and SIX3-R2. (Table 3). Thermal cycling conditions were as follows: initial denaturation: 2′ 94°C then 45″ 94°C, 45″ 64°C (for P12-F2/P12-R1), 59°C (for SIX3-F1/SIX3-R2) or 60°C (for SIX2-F2/SIX2-R2), 45″ 72°C, 30 cycles, and a final elongation step at 72°C for 10 min. Amplified products (1 μl) were resolved by gel electrophoresis in a 1.5% agarose gel. All reactions were performed at least twice.

Southern analysis

Southern analysis was performed on a subset of isolates from *F. oxysporum* (Fig. 2, Table 2). Genomic DNA was isolated according to the method of Raeder and Broda (Raeder and Broda, 1985) and digested with a combination of NcoI and XhoI restriction enzymes as described by the manufacturer (MBI Fermentas). Southern blotting was carried out according to (Sambrook *et al*., 1989) using (roughly) 10 μg of genomic DNA per lane. Probes were generated through PCR amplification: primers P12-F2B and P12-R1 (Table 3) were used to generate a 1 kb fragment containing the entire *SIX1* open reading frame (ORF). The *SIX2* ORF was amplified using SIX2-F1 and SIX2-R1 (Table 3), *FRP1* [F-box protein required for pathogenicity, used as control (Duyvesteijn *et al*., 2005)] was amplified with primers B442R7078 and B442F6250 (Table 3). Probes were radioactively labelled with [α-^{32}P]-dATP using the DecaLabelTM DNA labelling kit (MBI Fermentas). Hybridization was carried out overnight at 65°C in 0.5 m phosphate buffer, pH 7.2, containing 7% SDS and 1 mM EDTA. Blots were washed at 65°C with 0.5× SSC, 0.1% SDS. Hybridizing bands were visualized by phosphorimaging on a STORM 840 (Molecular Dynamics). Southern analysis was performed twice.

Sequencing and PCR amplification

For a few isolates used in our phylogenetic analyses the *EF-1*α (elongation factor 1α) and *mtSSU* rDNA (mitochondrial small subunit rDNA) sequences had never been determined. From these isolates *EF-1*α and *mtSSU* fragments were amplified and sequenced using genomic DNA as a template. Additionally, of the isolates causing tomato wilt *SIX1*, *SIX2* and an intergenic region were amplified and sequenced. For all fragments the PCR program was as follows: initial denaturation: 2′ 94°C, then 30″ 94°C, 45″ 50°C, 2′ 72°C, 40 cycles. Sequencing was performed directly on 1 μl of the PCR product, using the Big Dye Terminator kit (v1.1, Applied Biosystems). *SIX1* was amplified using primer P12-F2B and P12-R1, and sequenced using P12-F2B, P12-R1 and P12-R17 (Table 3). *SIX2* was amplified using primers SIX2-F2 and SIX2-R3, and sequenced using SIX2-F2, SIX2-R3 and P12-R12 (Table 3). *EF-1*α was amplified using primers EF1 and EF2B, and sequenced using EF1, EF11 and EF22 (Table 3). *mtSSU* was amplified using MS1 and MS2, and sequenced using MS21 and MS11B (Table 3). Part of the intergenic region between *SIX1* and *SHH1* was amplified using primers

Table 3. Primers used in this study.

Name	Sequence (5′-3′)	Target	Reference
P12-F1	ccccgaattgaggtgaag	*SIX1*	(Rep *et al.*, 2004)
P12-F2B	tatccctccggattttgagc	*SIX1*	–
P12-R1	aatagagcctgcaaagcatg	*SIX1*	(Rep *et al.*, 2004)
P12-R17	gaaagtggtgcactcctg	*SIX1*	–
P12-R12	cgtctaatcctacatctatg	*SIX2*	–
SIX2-F1	atgctcttcaaaatcgcgtg	*SIX2*	–
SIX2-F2	tctatccgctttcttctctc	*SIX2*	–
SIX2-R1	tcaacataggccacaccatt	*SIX2*	–
SIX2-R2	caacgccgtttgaataagca	*SIX2*	–
SIX2-R3	gtaaatagattgagctagcc	*SIX2*	–
SIX3-F1	ccagccagaaggccagttt	*SIX3*	–
SIX3-R2	ggcaattaaccactctgcc	*SIX3*	–
EF1	atgggtaagga(a/g)gacaagac	*EF-1α*	(O'Donnell *et al.*, 1998)
EF2B	ggaagtaccagtgatcatgtt	*EF-1α*	(O'Donnell *et al.*, 1998)
EF11	gtggggcatttaccccgcc	*EF-1α*	(O'Donnell *et al.*, 1998)
EF22	aggaacccttaccgagctc	*EF-1α*	(O'Donnell *et al.*, 1998)
TEF-F1	tcgtcgtcatcggccacgtc	*EF-1α*	–
TEF-R1	cgatgacggtgacatagtag	*EF-1α*	–
MS1	cagcagtcaagaatattagtcaatg	*mtSSU* rDNA	(White *et al.*, 1990)
MS2	gcggaattatcgaattaaataac	*mtSSU* rDNA	(White *et al.*, 1990)
MS21	ctctcctcctcaagtactgc	*mtSSU* rDNA	(White *et al.*, 1990)
MS11B	gcagtacttgaggaggag	*mtSSU* rDNA	(White *et al.*, 1990)
INTER-F1	agcagcacatctttcataac	intergenic region	–
INTER-R1	tggattgatacttttagccc	intergenic region	–
SIX1H-F5	gtgagaaggggcacaacg	*SIX1-H*	–
SIX1H-R7	ctttggatcgcaacttaattg	*SIX1-H*	–
SHH1-F1	tgactccccattatctcaatt	*SHH1*	–
SHH1-R1	tgttcacaaacatcatccgc	*SHH1*	–
B442R7078	gctagcttcgatcagctttt	*FRP1*	–
B442F6250	gtttcggtttcgacgttgac	*FRP1*	–
ITS4	tcctccgcttatt	rDNA	(White *et al.*, 1990)
ITS5	ggaagtaaaagtcgtaacaagg	rDNA	(White *et al.*, 1990)

INTER-F1 and INTER-R1, and sequenced with the same primers (Table 3).

The PCR products shown in Fig. 2 were obtained using the same genomic DNA samples, using 1 μl as a template in a 25 μl PCR reaction, subjected to the following program: initial denaturation: 2′ 94°C, then 30″ 94°C, 45″ 50°C, 2′ 72°C, 35 cycles. The following primers were used: *SIX1*: P12-F1 and P12-R1, *SIX2*: SIX2-F1 and SIX2-R1, *EF-1α*: TEF-F1 and TEF-R1, *SIX1-H*: SIX1H-F5 and SIX1H-R7, *SHH1*: SHH1-F1 and SHH1-R1, *SIX3*: SIX3-F1 and SIX3-R2 (Table 3).

Phylogenetic analyses

Phylogenetic analyses were performed on DNA sequences of the *EF1-α* gene and *mtSSU* rDNA. A neighbour-joining tree was constructed using MacVector (Oxford Molecular Group) on the combined data set of both genes for a selection of 19 *F. oxysporum* isolates. Clade stability was assessed by 1000 bootstrap replications. The outgroup species used for rooting the tree was used previously as an outgroup to a similar set of isolates by (O'Donnell *et al.*, 1998) and represents a putative sister group to the *F. oxysporum* complex (O'Donnell and Cigelnik, 1997).

Fusarium *transformation*

Fol was transformed with *Agrobacterium*-mediated transformation, as described previously (Takken *et al.*, 2004). Briefly, 10^5 fungal spores were mixed with the same volume of an *Agrobacterium tumefaciens* suspension ($OD_{660} = 0.45$) in induction medium [IM: 10 mM glucose, 10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 4 mM $(NH_4)_2SO_4$, 0.7 mM $CaCl_2$, 2 mM $MgSO_4$, 9 mM $FeSO_4$, 0.5% (w/v) glycerol, 910 ml of 40 mM MES, pH 5.3] supplemented with 200 mM acetosyringone. The mixture was transferred to filters (ME25; Schleicher and Schuell) on co-cultivation plates (composition as IM, but with 5 mM glucose and 1.5% agar). Plates were incubated at 25°C for 2 days. Transformants were selected by transfer of the filters to Czapek Dox agar (CDA, Oxoid) with cefotaxime (100 mg ml^{-1}, Duchefa) and zeocin (100 mg ml^{-1}, Invivo-Gen). Media containing zeocin were buffered with 0.1 M Tris, pH 8. Of every transformation, four independent transformants were tested in a bioassay.

Construction of a binary vector for expression of SIX1

The binary vector for expression of *SIX1* was designed in such a way that it could be used for both ectopic insertion and recombination at the original *SIX1* locus. The HindIII-SpeI fragment containing the *SIX1* gene from Fol isolate Fol004 (Rep *et al.*, 2005a) in the pGEMT-easy vector, was extended at the 3′ end with the neighbouring 1.7 kb SpeI-XbaI DNA fragment from a BAC clone. To allow selection for transformants in *Fusarium* the *ble* resistance gene (conferring resistance to zeocin) was introduced downstream of the *SIX1* terminator. For this, the *ble* gene was retrieved from the pAN8.1 vector (Punt and van den Hondel, 1992) as an

AgeI-XbaI fragment and cloned into the SgrAI-SpeI digested *SIX1* locus in pGEMT-easy. HindIII and EcoRI were used to transfer the expression cassette to pPZP200, a binary vector for *Agrobacterium*-mediated tranformation of *Fusarium* (Hajdukiewicz *et al*., 1994).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Transformation of the *SIX1* gene to non-*lycopersici* isolates does not lead to the ability to cause tomato wilt disease. (A) PCR on genomic DNA of independant transformants (A–E) using primers annealing to the *SIX1* ORF [P12-F1, P12-R1 (Table 3)]. (B) The different transformants are unable to cause tomato wilt. Tomato seedlings were inoculated with a Fol isolate or independent transformants of Fom, Forl or Fo-47. Disease was measured three weeks after inoculation. The bars indicate the average disease index (DI) of 10 plants, with error bars indicating the 95% confidence interval. DI was measured in arbitrary units (0, no symptoms; 1, slightly swollen and/or bent hypocotyl; 2, one or two brown vascular bundles in hypocotyl; 3, at least two brown vascular bundles and growth distortion; 4, all vascular bundles are brown, plant either dead or very small and wilted). Fol, f.sp. *lycopersici* isolate Fol 007; Fom, f.sp. *melonis* isolate 26406; Forl, f.sp. *radicis-lycopersici* isolate Forl 723; Fo-47, a non-pathogenic *Fo* isolate (see Table 2 for more detailed descriptions of these isolates).

Table S1. Isolates of *Fusarium oxysporum* and other *Fusarium* sp. used in this study.

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