Related mobile pathogenicity chromosomes in Fusarium oxysporum determine host range on cucurbits

Li, J.; Fokkens, L.; van Dam, P.; Rep, M.

DOI
10.1111/mpp.12927

Publication date
2020

Document Version
Final published version

Published in
Molecular Plant Pathology

Received: 1 August 2019 | Revised: 24 January 2020 | Accepted: 5 February 2020

DOI: 10.1111/mpp.12927

ORIGINAL ARTICLE

Molecular Plant Pathology | WILEY

Related mobile pathogenicity chromosomes in *Fusarium oxysporum* determine host range on cucurbits

Jiming Li [1] | Like Fokkens[1] | Peter van Dam[2] | Martijn Rep [1]

[1]Molecular Plant Pathology, University of Amsterdam, Amsterdam, Netherlands

[2]Genetwister Technologies B.V., Wageningen, Netherlands

Correspondence
Martijn Rep, Molecular Plant Pathology, University of Amsterdam, Amsterdam, 1098 XH, Netherlands.
Email: m.rep@uva.nl

Funding information
J.L. was financially supported by the China Scholarship Council program (file number: 201504910768). L.F. was financially supported by the NWO Talent Scheme Veni (grant number: 016.veni.181.090).
No conflict of interest is declared.

Abstract

Fusarium oxysporum f. sp. *radicis-cucumerinum* (Forc) causes severe root rot and wilt in several cucurbit species, including cucumber, melon, and watermelon. Previously, a pathogenicity chromosome, chr^{RC}, was identified in Forc. Strains that were previously nonpathogenic could infect multiple cucurbit species after obtaining this chromosome via horizontal chromosome transfer (HCT). In contrast, *F. oxysporum* f. sp. *melonis* (Fom) can only cause disease on melon plants, even though Fom contains contigs that are largely syntenic with chr^{RC}. The aim of this study was to identify the genetic basis underlying the difference in host range between Fom and Forc. First, colonization of different cucurbit species between Forc and Fom strains showed that although Fom did not reach the upper part of cucumber or watermelon plants, it did enter the root xylem. Second, to select candidate genomic regions associated with differences in host range, high-quality genome assemblies of Fom001, Fom005, and Forc016 were compared. One of the Fom contigs that is largely syntenic and highly similar in sequence to chr^{RC} contains the effector gene *SIX6*. After HCT of the *SIX6*-containing chromosome from Fom strains to a nonpathogenic strain, the recipient (HCT) strains caused disease on melon plants, but not on cucumber or watermelon plants. These results provide strong evidence that the differences in host range between Fom and Forc are caused by differences between transferred chromosomes of Fom and chr^{RC}, thus narrowing down the search for genes allowing or preventing infection of cucumber and watermelon to genes located on these chromosomes.

KEYWORDS

cucurbits, fungal colonization, *Fusarium oxysporum*, horizontal chromosome transfer, host range, pathogenicity chromosomes

1 | INTRODUCTION

Fusarium oxysporum (Fo) is a soilborne fungus and is very common worldwide (Di Pietro *et al.*, 2003). Most Fo strains are not pathogenic and some are known to be beneficial to plants (Di Pietro *et al.*, 2003; Bubici *et al.*, 2019). However, as a species complex, Fo can cause severe disease on more than 100 plant species, including some economically important crops such as chickpea, tomato, banana, and cucurbits (Michielse and Rep, 2009). Individual strains of Fo can only infect one or a few related plant species (Kistler, 1997). Based on this

host specificity, pathogenic Fo strains are classified into different *formae speciales* (ff. sp.). For example, Fo f. sp. *radicis-cucumerinum* (Forc) causes disease on the cucurbit species cucumber, melon, and watermelon, while Fo f. sp. *melonis* (Fom) can only cause disease on melon. The genetic basis underlying the difference in host range between Fom and Forc is still unknown.

To cause disease Fo first penetrates the root epidermis and then grows intercellularly or intracellularly in the root cortex. Eventually the fungus reaches the xylem vessels of the roots and then spreads to the main root and stem in susceptible plants (Michielse and Rep, 2009). In resistant plants, however, the fungus is mostly restricted to epidermal cells and the cortex (Upasani *et al*., 2016). Colonization patterns of compatible or incompatible interactions between Fo and its host plants has been investigated in several ff. sp., including f. sp. *melonis* (Zvirin *et al*., 2010), f. sp. *ciceris* (Jimenez-Fernandez *et al*., 2013; Upasani *et al*., 2016), f. sp. *conglutinans* (Li *et al*., 2014), f. sp. *radicis-cucumerinum* (Cohen *et al*., 2015), f. sp. *phaseoli* (Niño-Sánchez *et al*., 2015; Garces-Fiallos *et al*., 2017), f. sp. *niveum* (Zhang *et al*., 2015), and f. sp. *pisi* (Bani *et al*., 2018). However, to our knowledge, colonization differences between host and nonhost plants by Fo have not been studied so far.

To colonize plants, pathogens secrete a variety of molecules, including cell wall-degrading enzymes, secondary metabolites, and small proteins called effectors (Perez-Nadales *et al*., 2014). Effectors have been shown to contribute to the virulence of xylem-colonizing fungal pathogens including Fo (de Sain and Rep, 2015), *Verticillium* (de Jonge *et al*., 2012, 2013), *Ceratocystis*, and *Ophiostoma* (de Sain and Rep, 2015). For example, in Fo f. sp. *lycopersici* (Fol), Six1 (Secreted in xylem 1) (Rep *et al*., 2004), Six3 (Houterman *et al*., 2009), and Six5 (Ma *et al*., 2015) have been shown to contribute to virulence. Some effectors also act as avirulence factors that can be recognized by host immune receptors (Rep *et al*., 2004; Houterman *et al*., 2008; 2009; de Jonge *et al*., 2012).

In filamentous plant pathogens, virulence genes are commonly located in distinct genomic regions (Croll and McDonald, 2012). These chromosomal regions or chromosomes are often rich in transposable elements (TEs) and undergo relatively fast evolution compared to the rest of the genome (Croll and McDonald, 2012). For example, in Fol all identified *SIX* genes are located on one "pathogenicity" chromosome, which is essential for infection (Ma *et al*., 2010). TEs occupy at least 24% of this chromosome – twice as much as non-TE genes (Schmidt *et al*., 2013). In Forc, some *SIX* homologs are present in a region of a horizontally transferrable pathogenicity chromosome that is exceptionally TE-rich (van Dam *et al*., 2017).

In several fungal plant pathogens, virulence genes or entire chromosomes can undergo horizontal transfer and thereby confer host-specific pathogenicity (Friesen *et al*., 2006; Akagi *et al*., 2009; Ma *et al*., 2010; Mehrabi *et al*., 2011). In Fo, mobile pathogenicity chromosomes have been identified for tomato-infecting and cucurbit-infecting strains (Ma *et al*., 2010; Vlaardingerbroek *et al*., 2016a; van Dam *et al*., 2017). In cucurbit-infecting Forc strains, the pathogenicity chromosome chr^{RC} that determines root and shoot rot was identified recently and shown to be largely syntenic with a contig in the genome assembly of melon-infecting strain Fom001 (van Dam *et al*., 2017). We hypothesized that this contig in Fom001 determines virulence towards melon plants, and differences between chr^{RC} and this contig determine differences in host range between Forc and Fom. Furthermore, we hypothesized that if this contig determines virulence towards melon, it is also present in Fom strains other than Fom001, hence we included Fom005 (vegetative compatibility group [VCG] 0134), which does not belong to the same clonal line as Fom001 (VCG 0136) (Schmidt *et al*., 2016; van Dam *et al*., 2017).

In this study, we set out to (a) investigate colonization of different cucurbit species by green fluorescent protein (GFP)-labelled Forc and GFP-labelled Fom strains, (b) identify pathogenicity chromosomes in two Fom strains, and (c) identify genomic regions or chromosomes that contribute to differences in host range between Fom and Forc.

2 | RESULTS

2.1 | Forc and Fom can colonize xylem vessels of cucumber, melon, and watermelon roots

Forc causes disease on multiple cucurbit plant species, while Fom only causes disease on melon plants (van Dam *et al*., 2016). We hypothesized that Forc strains fully colonize cucumber and watermelon plants, while colonization by Fom strains is restricted in cucumber and watermelon roots. Fungal biomass in cucumber and melon roots 1 week after inoculation with Forc016, Fom001 or Fom005 was quantified by quantitative PCR (qPCR). The phenotypes of respective cucumber and melon plants are shown in Figure S1. The identical single-copy gene *SIX6* in Fom and Forc was selected to quantify fungal biomass, while the *α-tubulin* gene of cucumber or melon was used for normalization to plant DNA. As expected, in the infected cucumber roots the biomass of Fom001 and Fom005 was about 20 times less than the biomass of Forc016 (Figure 1a). In the infected melon roots, no significant fungal biomass differences were observed between these three strains (Figure 1b). The biomass differences between Fom001 and Fom005 were very small (Figure 1), therefore only Fom005 and Forc016 were used in the remaining experiments described in this section.

To further assess potential differences in colonization of cucumber, melon, and watermelon roots by Forc and Fom, Forc016 and Fom005 transformants carrying *GFP* under a constitutive promoter (*FEM1* promoter), which showed wild-type virulence on cucumber and melon, were selected to inoculate cucumber, melon, and watermelon seedlings on Petri dishes. Colonization of infected cucumber and melon roots was then observed microscopically at 6 days post-inoculation (dpi) and at 16 dpi. Both Forc016_GFP and Fom005_GFP reached xylem vessels of cucumber and melon roots at 6 dpi. However, further growth of Fom005_GFP hyphae into the hypocotyl of cucumber was not observed, and these plants still appeared healthy (data not shown).

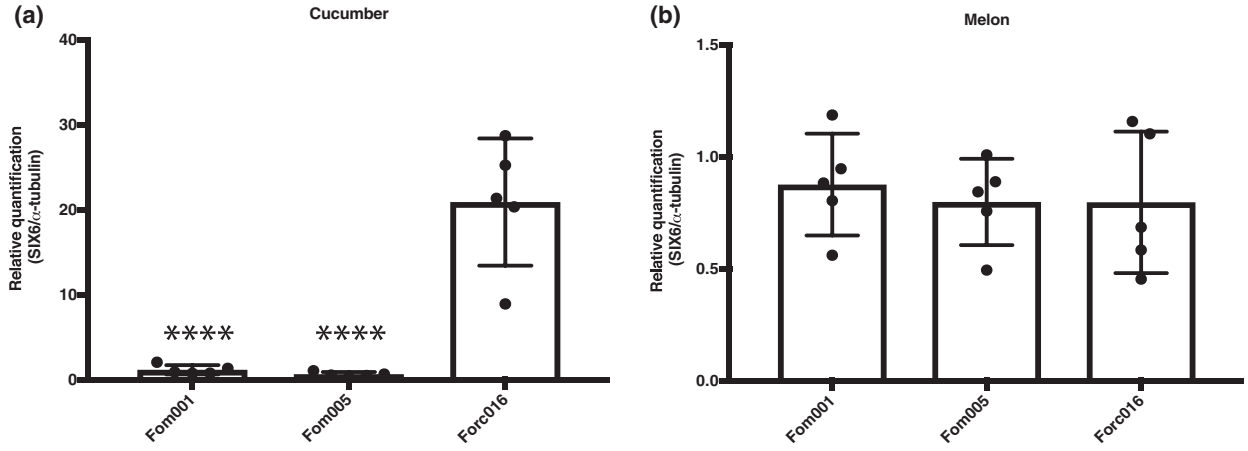

FIGURE 1 Fom001 and Fom005 are restricted in cucumber roots. Fungal biomass of Fom001, Fom005, and Forc016 in cucumber and melon roots was quantified using quantitative PCR 1 week after infection. The single-copy gene *SIX6* in *Fusarium oxysporum* f. sp. *melonis* (Fom) and *F. oxysporum* f. sp. *radicis-cucumerinum* (Forc) was used to quantify fungal DNA, while the *α-tubulin* gene of cucumber or melon was used for normalization to plant DNA. Five biological replicates and three technical replicates were used for each treatment. RQ, relative quantification of fungal biomass; the lowest sample was given a value of 1 and the rest were calculated relative to that value. The asterisks (****) indicate significantly less fungal biomass when compared with the biomass of Forc016 (one-way analysis of variance test, $p < .0001$). (a) In cucumber roots, the fungal biomass of Fom001 and Fom005 was about 20 times less than the biomass of Forc016. (b) In melon roots, no significant biomass differences were observed between these three strains

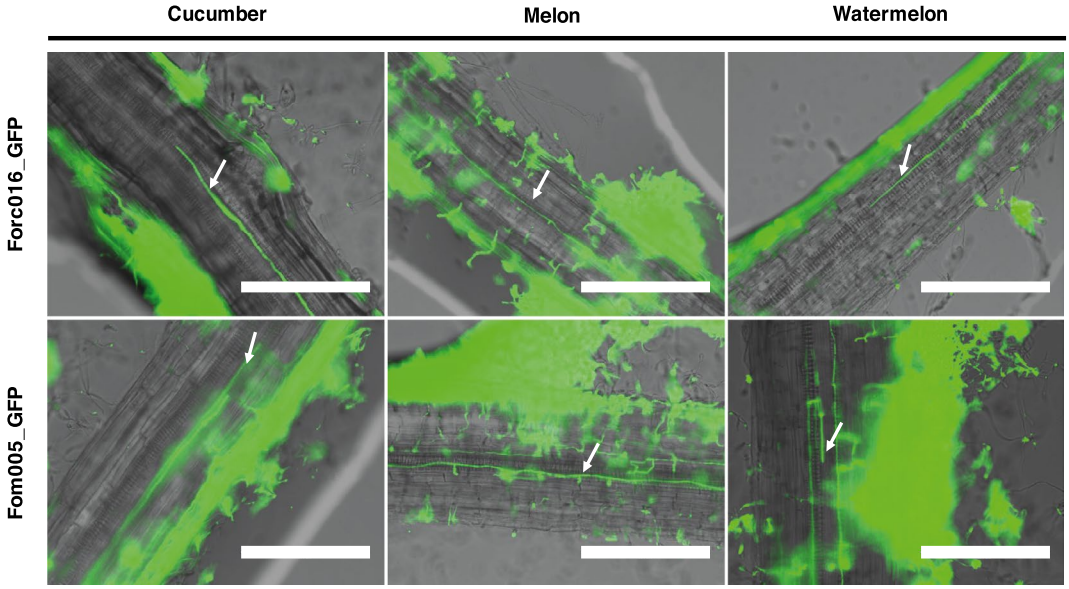

FIGURE 2 Forc016 and Fom005 can colonize xylem vessels of cucumber, melon, and watermelon at 3–4 days post-inoculation (dpi). Six-day-old cucumber and 10-day-old melon and watermelon seedlings were treated with water (not shown), Forc016_GFP (10^7 spores/ml), and Fom005_GFP (10^7 spores/ml) in Petri dishes. At 3–4 dpi, xylem colonization of all plants by both Forc016_GFP and Fom005_GFP was observed. The white arrows indicate hyphae growing inside xylem vessels. The white bars are 200 μm in length

To investigate colonization differences before 6 dpi in more detail, infected roots of cucumber, melon, and watermelon plants were observed microscopically at 1, 2, 3, 4, and 14 dpi using the same methods mentioned above. At 1 dpi both Forc016_GFP and Fom005_GFP spores had germinated and some hyphae were present intercellularly between epidermal cells of all infected plants, while the plants appeared to be healthy. At 2 dpi hyphae were present extensively in the root cortex of all infected plants. At this stage, while no colonization of the xylem was observed, root browning in all infected plants was apparent. During 3–4 dpi (Figure 2), xylem colonization for all infected roots was observed and all roots were dark brown. Disease progress was followed until 14 dpi, at which time Forc016_GFP-infected cucumber, melon, and watermelon plants all showed severe wilting, and root and stem rot (Figure S2). Fom005_GFP-infected melon plants showed wilting, and root and stem rot, while cucumber and watermelon plants infected with

Fom005_GFP still appeared healthy with light brown roots (Figure S2). To assess whether Fom005_GFP colonized the upper part of healthy-appearing cucumber and watermelon plants at 14 dpi, the presence of Fom005_GFP hyphae at the hypocotyl level was assessed. No hyphae or spores were found at the hypocotyl level for both cucumber (Figure S3) and watermelon plants (data not shown).

Interestingly, at 3–4 dpi, we observed that cucumber roots started to produce new roots. This was not the case for melon and watermelon. At 14 dpi, Fom005_GFP-infected cucumber plants still appeared healthy with a large number of newly produced, thick lateral roots (Figure S2), while Forc016_GFP-infected cucumber plants had fewer and shorter newly produced lateral roots (Figure S2). In addition, Forc016_GFP-infected cucumber showed severe root rot within 2 weeks. Fom005_GFP-infected watermelon plants showed stress symptoms (leaves turning from shiny to dull) during 3–4 dpi but they were fully recovered at 14 dpi; production of new roots was not observed. In addition, watermelon had fewer roots than cucumber and melon, but watermelon roots were much thicker than cucumber and melon roots.

To conclude, like Forc, melon-infecting strains can enter the root xylem of cucumber and watermelon plants without reaching the stem in the hydroponic infection system employed.

2.2 | Part of the genome of Fom001 and Fom005 is syntenic with the pathogenicity chromosome in Forc

Previously, a mobile pathogenicity chromosome, called chr^{RC}, was identified in Forc016 (van Dam *et al.*, 2017). Comparisons of chr^{RC} to a high-quality assembly of Fom001 revealed that the genome of Fom001 contains two contigs, contig 127 and contig 13, that are largely syntenic with chr^{RC} (van Dam *et al.*, 2017) (Figure 3a). These two contigs have an overlap of around 10 kb but with some single nucleotide polymorphisms in the overlap region. If these two contigs constitute a pathogenicity chromosome in Fom (chr^{MLN}), syntenic contigs were expected to be present in a genome assembly of Fom005. The genome of Fom005 was sequenced with single molecule real-time (SMRT) sequencing to achieve a high-quality genome assembly (Table S1). This assembly was compared to the putative pathogenicity chromosome contigs of Fom001 and the previously identified chr^{RC} of Forc016. We found that in the genome assembly of Fom005, chr^{RC}-syntenic contigs include contigs 16, 139, 129, 168, and 144 (Figure 3b). No end-overlap was found among these contigs. Interestingly, chr^{RC}-syntenic regions in Fom001 and Fom005 are almost 100% identical (Figure 3c). Two single-copy candidate effector genes, *SIX6* and *SIX11*, are located in the repeat-rich region of

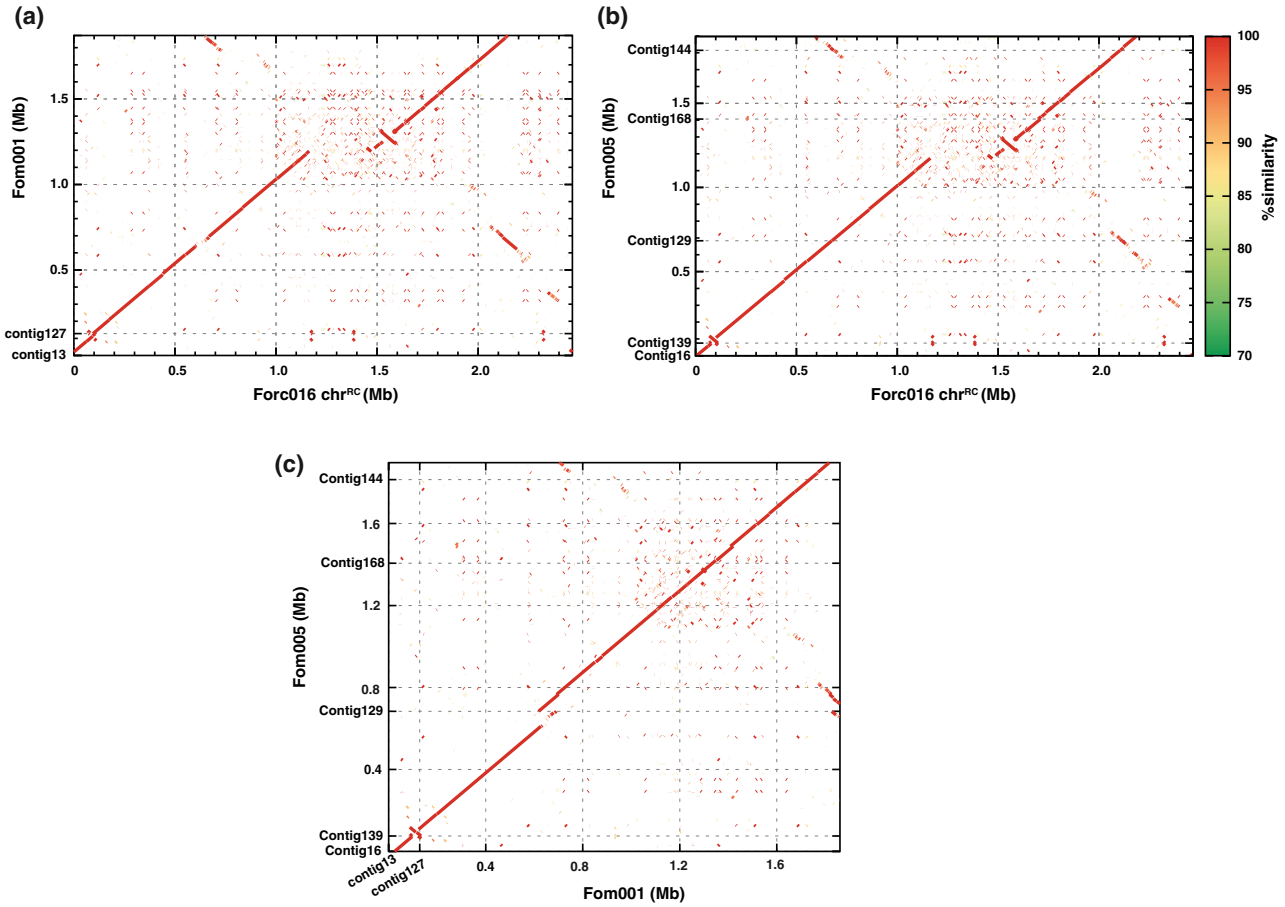

FIGURE 3 chr^{RC} syntenic regions are present in Fom001 and Fom005. (a) Contigs 13 and 127 of Fom001 are largely syntenic with Forc016 chr^{RC} (nucmer alignment with manual adjustment). These syntenic regions are also present in Fom005 and include contigs 16, 139, 129, 168, and 144 (b). Contigs 13 and 127 of Fom001 are almost identical to contigs 16, 139, 129, 168, and 144 of Fom005 (c). Alignments are colour-coded according to their similarity

Fom001 contig 127 and Fom005 contig 129. Both of these *SIX* gene homologs are identical to the *SIX6* and *SIX11* homologs present on chr^{RC}. We hypothesized that the chr^{RC}-syntenic contigs in Fom001 and Fom005 constitute a pathogenicity chromosome containing virulence genes for infection of melon.

2.3 | Horizontal transfer of Fom chromosomes

If the putative pathogenicity chromosome (chr^{MLN}) that we identified in Fom001 and Fom005 is sufficient to enable melon infection, transfer of this chromosome into a nonpathogenic strain was expected to render the recipient strain pathogenic on melon. To test this, a hygromycin-resistance gene (*HYG*) as a marker was placed in the *SIX6* locus to select for transfer of chr^{MLN} from Fom001 and Fom005 to a nonpathogenic strain. Two Fom001-derived strains (Fom001ΔSIX6#11 and Fom001ΔSIX6#34) and three Fom005-derived strains (Fom005ΔSIX6#1, Fom005ΔSIX6#17, and Fom005ΔSIX6#87) were obtained in which *SIX6* was replaced with *HYG*. When melon plants were inoculated with these *SIX6* deletion strains, no reduction of virulence was observed compared to the wild-type strains (Figure S4), indicating that *SIX6* does not contribute to virulence of Fom under the tested conditions.

To test the transferability of *HYG*-labelled chr^{MLN} from Fom001 and/or Fom005, three potential hygromycin-resistant donor strains, Fom001ΔSIX6#11, Fom005ΔSIX6#1, and Fom005ΔSIX6#87, were co-incubated with the nonpathogenic strain Fo47 containing a randomly inserted zeocin-resistance marker (Vlaardingerbroek *et al.*, 2016a). As positive control, Forc016ΔSIX6#46 was included as a donor strain (van Dam *et al.*, 2017). Double drug-resistant colonies were found for all combinations (Table 1). Ten double drug-resistant colonies were recovered from co-incubating Fom001ΔSIX6#11 with Fo47, and two double drug-resistant colonies were found by co-incubating Fom005ΔSIX6#1 and Fom005ΔSIX6#87 with Fo47, respectively. For Forc016ΔSIX6#46, only one double drug-resistant colony was obtained. All double drug-resistant colonies were confirmed by PCR to have a Fo47-specific SCAR marker and to carry both *HPH* and *BLE* genes.

TABLE 1 Horizontal chromosome transfer (HCT) strains

Donor strain	HCT strain
Fom001ΔSIX6#11	Fom001_HCT#1
	Fom001_HCT#2
	Fom001_HCT#3
	Fom001_HCT#4
	Fom001_HCT#5
	Fom001_HCT#6
	Fom001_HCT#7
	Fom001_HCT#8
	Fom001_HCT#9
	Fom001_HCT#10
Fom005ΔSIX6#1	Fom005_HCT#1
	Fom005_HCT#2
Fom005ΔSIX6#87	Fom005_HCT#3
	Fom005_HCT#4
Forc016ΔSIX6#46	Forc016_HCT#1

Note.The recipient strain is Fo47 in all cases.

To assess horizontal chromosome transfer (HCT) and visualize the karyotypes of the putative HCT strains, Fom001_HCT #3, #4, #5, Fom005_HCT #1, #2, and Forc016_HCT#1 (Table 1) were selected for contour-clamped homogeneous electric field (CHEF) electrophoresis analysis. All HCT strains showed the karyotype pattern of Fo47, with extra chromosomes visible in some strains (Figure 4). Surprisingly, two chromosomes were transferred from Fom005ΔSIX6#1 to Fo47 for both HCT strains tested. These two HCT strains may have originated from the same transfer event as they emerged on the same double-selective plate. For HCT strains Fom001_HCT #3, #4, and #5, no extra band was observed. In the case of Forc016_HCT#1, chr^{RC} comigrated with the smallest chromosome of Fo47, which showed a band with double intensity on the gel, as was also observed in the previous study (van Dam *et al.*, 2017).

To investigate which sequences of the Fom001 and Fom005 genomes had been transferred to Fo47, the genomes of three Fom001 HCT strains, Fom001_HCT #3, #4, #5, and three Fom005 HCT strains, Fom005_HCT#2, #3, #4 were sequenced. In addition, the genome of the recipient strain Fo47 was also sequenced with SMRT sequencing (Table S1) and used this assembly in downstream analyses. To identify which chromosomes were newly acquired during the transfer experiment, all reads that did not map to the genome of the recipient strain Fo47 were extracted, and these reads were mapped to the genome assembly of the donor strain Fom001 (Figure 5) or Fom005 (Figure 6). Figure 5a,b shows that, even though no extra band was observed on the CHEF gel, HCT strains Fom001_HCT #3 and #4 resulted from transfer of Fom001 contigs 124 (c.2.1 Mb), 3 (c.1.5 Mb), 34 (c.0.07 Mb), 13 (c.0.1 Mb), and 127 (c.1.7 Mb) to Fo47. The HCT strain Fom001_HCT #5 resulted from transfer of part of contig 124, part of contig 3, and the entire contigs 13, 34, and 127. It was shown earlier that the size of the *SIX6*-containing chromosome in Fom001 is around 2 Mb (van Dam *et al.*, 2017). Therefore, contigs 127 and 13 most likely constitute chr^{MLN}, which is largely syntenic with chr^{RC}, while contig 124, contig 3, and contig 34 could form one chromosome or belong to different chromosomes. Part of the read densities of contig 127 were twice as high as those of the rest of the contig in all three HCT strains.

HCT strains Fom005_HCT#2, #3, and #4 all resulted from transfer of contigs 17 (1.6 Mb), 134 (c.0.2 Mb), 140 (c.0.2 Mb), 41 (c.0.1 Mb), 29 (c.0.1 Mb), 16 (c.0.1 Mb), 139 (c.0.6 Mb), 129 (c.0.7 Mb), 168 (c.0.4 Mb), 144 (c.0.1 Mb), and 142 (c.0.15 Mb) to Fo47 (Figure 6, contigs in bold [c.2 Mb] are syntenic with chr^{RC}). Read densities of contigs 16, 139, 129, 168, 144, and 142 were twice as high as those of contigs 17, 134, 140, 41, and 29 (Figure 6, contigs in bold are syntenic with chr^{RC}). Because two chromosomes, both of around 2 Mb in size, were observed to be transferred from Fom005 to Fo47

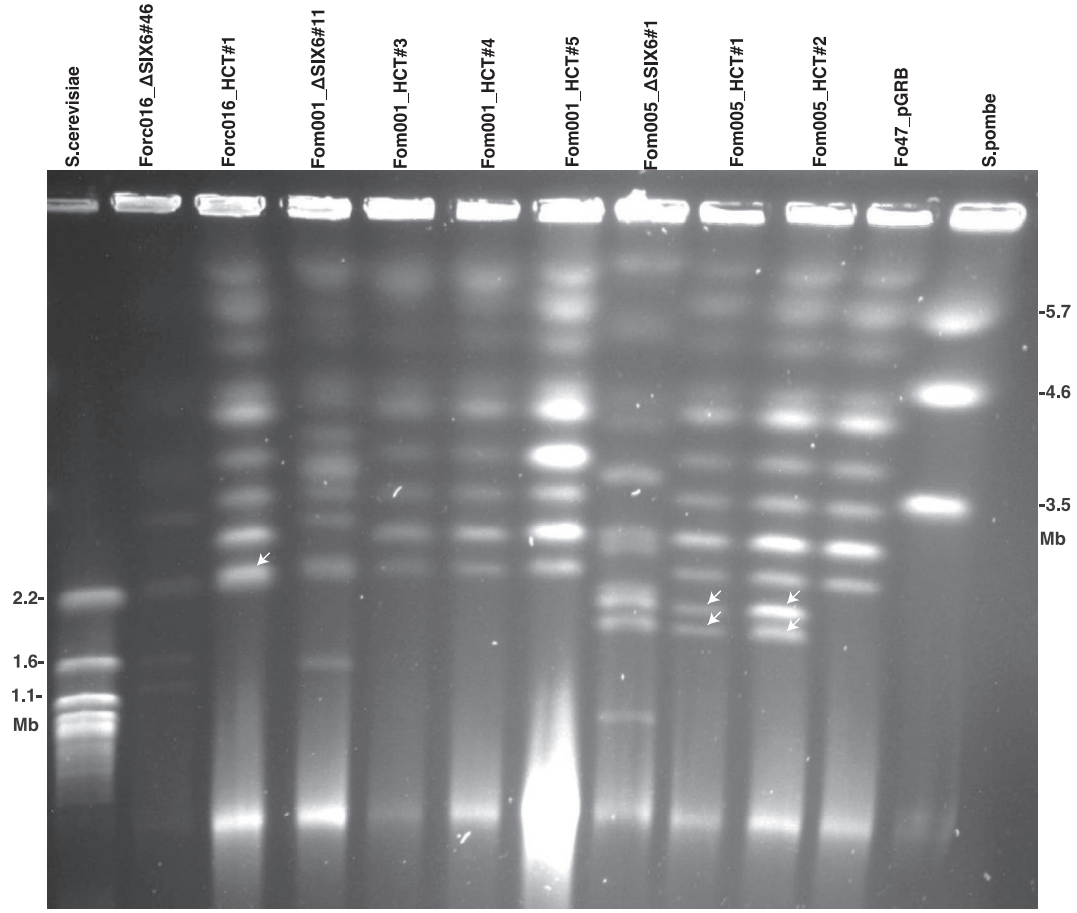

FIGURE 4 Contour-clamped homogeneous electric field electrophoresis revealed transfer of two chromosomes from Fom005ΔSIX6#1 to Fo47_pGRB. Horizontal chromosome transfer strains Forc016_HCT#1, Fom001_HCT #3, #4, #5, and Fom005_HCT #1, #2 have electrophoretic karyotypes similar to that of Fo47_pGRB. In addition, strains Fom005_HCT #1 and #2 have two extra bands (indicated by arrows), corresponding to bands of similar size present in the donor strain Fom005ΔSIX6#1. No extra band is visible in the karyotypes of Fom001_HCT #3, #4, and #5. A double band (indicated by arrows) is present in Forc016_HCT#1 at the position of the smallest chromosome of Fo47_pGRB. The karyotype of the donor strain Forc016 ΔSIX6#46 is weakly visible due to a low amount of DNA loaded. Chromosomes of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* were used as markers

(Figure 4) and the contigs with higher read densities were syntenic to chr^{RC} (Figure 3), we speculate that the contigs with higher read densities together form one transferred chromosome and the contigs with lower read densities form the other chromosome. In conclusion, all Fom001 and Fom005 HCT strains had gained extra sequences besides the chr^{RC} syntenic regions. These extra sequences are not syntenic between the Fom001 and Fom005 HCT strains (Figure S5).

To determine whether core chromosomes may have been transferred to HCT strains (Vlaardingerbroek *et al.*, 2016a), reads of the HCT strains were mapped directly to the SMRT assembly of the donor strains Fom001 or Fom005, and only those reads that mapped completely and without any mismatches were selected. In the case of transfer of core chromosomes, a high density of perfectly mapped reads was expected, even in the subtelomeric regions. However, this was not observed (Figure S6a–d) and thus we concluded that no core chromosomes had been transferred. This result was corroborated by the fact that the band pattern of the HCT strains on the CHEF gel was more similar to that of the recipient strain (Figure 4). In addition, no reads of the HCT strains mapped to part of contig 10 and the complete contig 22 in Fom001 (Figure S6a), indicating that these regions belong to the accessory (nonconserved) genome of Fom001 and were not transferred. Interestingly, a *SIX11* homolog and a *SIX13* homolog are located on this part of contig 10, while a *SIX1* homolog is located on contig 22. Contigs 4 and 23 of Fom005 are also absent in Fo47 (Figure S6c), but no *SIX* gene homologs are present on these contigs. A *SIX1* homolog, a *SIX11* homolog, and a *SIX13* homolog, which are located on contig 8 of Fom005, were also not transferred.

To check whether contigs 129, 139, 168, 144, 142, and 16 of Fom005 have duplicated in the HCT strains, or whether these apparently duplicated regions had been collapsed during assembly of the Fom005 genome, previously generated Fom005 reads (Schmidt *et al.*, 2016) and HCT strains Fom005_HCT#2, #3, and #4 were mapped to the SMRT assembly of Fom005. If these large-scale duplicated regions had collapsed during assembly of Fom005, higher read densities of Fom005 in these regions were expected compared

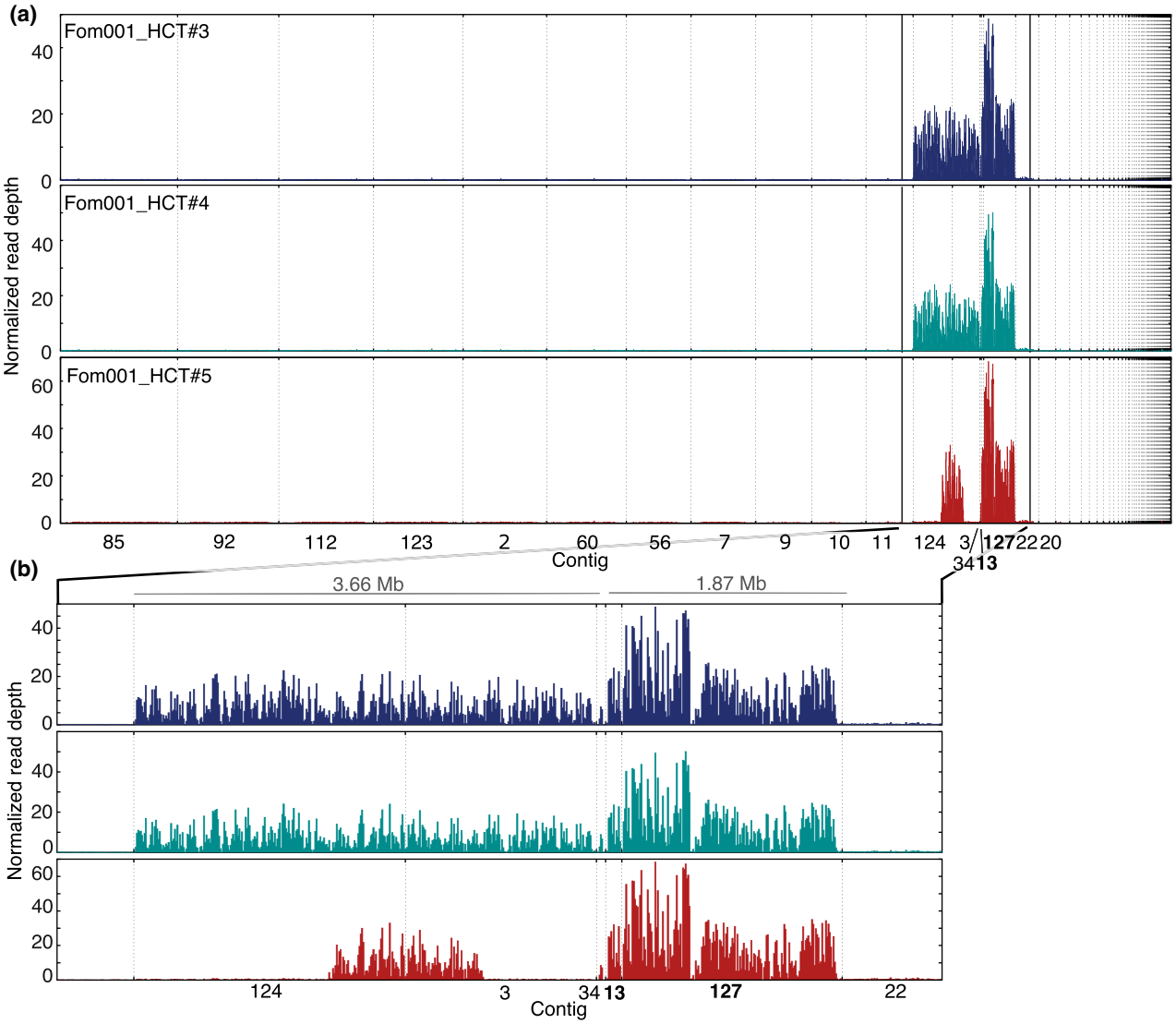

FIGURE 5 Normalized Illumina read mapping confirms horizontal chromosome transfer (HCT) from Fom001 to Fo47 and reveals partial deletions. Reads of HCT strains were first mapped to Fo47. Read-pairs that were not mapped to Fo47 were extracted and these unmapped reads were mapped to the single molecule real-time (SMRT) sequencing assembly of Fom001 (a). HCT strains Fom001_HCT #3 (blue line) and #4 (green line) resulted from transfer of Fom001 contigs 124 (c.2.1 Mb), 3 (c.1.5 Mb), 34 (c.0.07 Mb), 13 (c.0.1 Mb), and 127 (c.1.7 Mb) to Fo47. The HCT strain Fom001_HCT #5 (red line) resulted from transfer of part of contigs 124 and 3, and the whole of contigs 34, 13, and 127 (b). Read densities were normalized by dividing by average read density

to other regions. We did not observe this, however, and thus concluded that these regions had duplicated in the HCT strains derived from Fom005 (Figure S6e). Clear read density differences between HCT strains and the donor strain were observed (dark blue line) (Figure S6e), confirming that no core chromosomes had been transferred into Fom005 HCT strains.

2.4 | Horizontal transfer of Fom chromosomes can turn a nonpathogen into a strain that can infect melon, but not cucumber or watermelon

To test whether chromosomes transferred from Fom strains are sufficient for infecting melon, bioassays on susceptible melon plants were performed, comparing Fom001_HCT #3, #4, #5, Fom005_HCT #1, #2, #3, #4, and Forc016_HCT#1 to donor strains Fom001, Fom005, Forc016, and mock treatment. All donor strains and HCT strains caused disease on melon plants, but there were differences in the severity of disease symptoms observed (Figure 7a). The three Fom001_HCT strains caused only moderate disease symptoms on melon plants compared to their donor strain (Figure 7a). In contrast, melon plants infected with each of the four Fom005_HCT strains showed severe disease symptoms, comparable to infection with the respective donor strains. To conclude, the chromosomes transferred from Fom001 and Fom005 determine virulence on melon plants (Figure 8a,b).

We considered that the difference in host range between Fom and Forc could be caused by (a) avirulence genes present in Fom (but absent

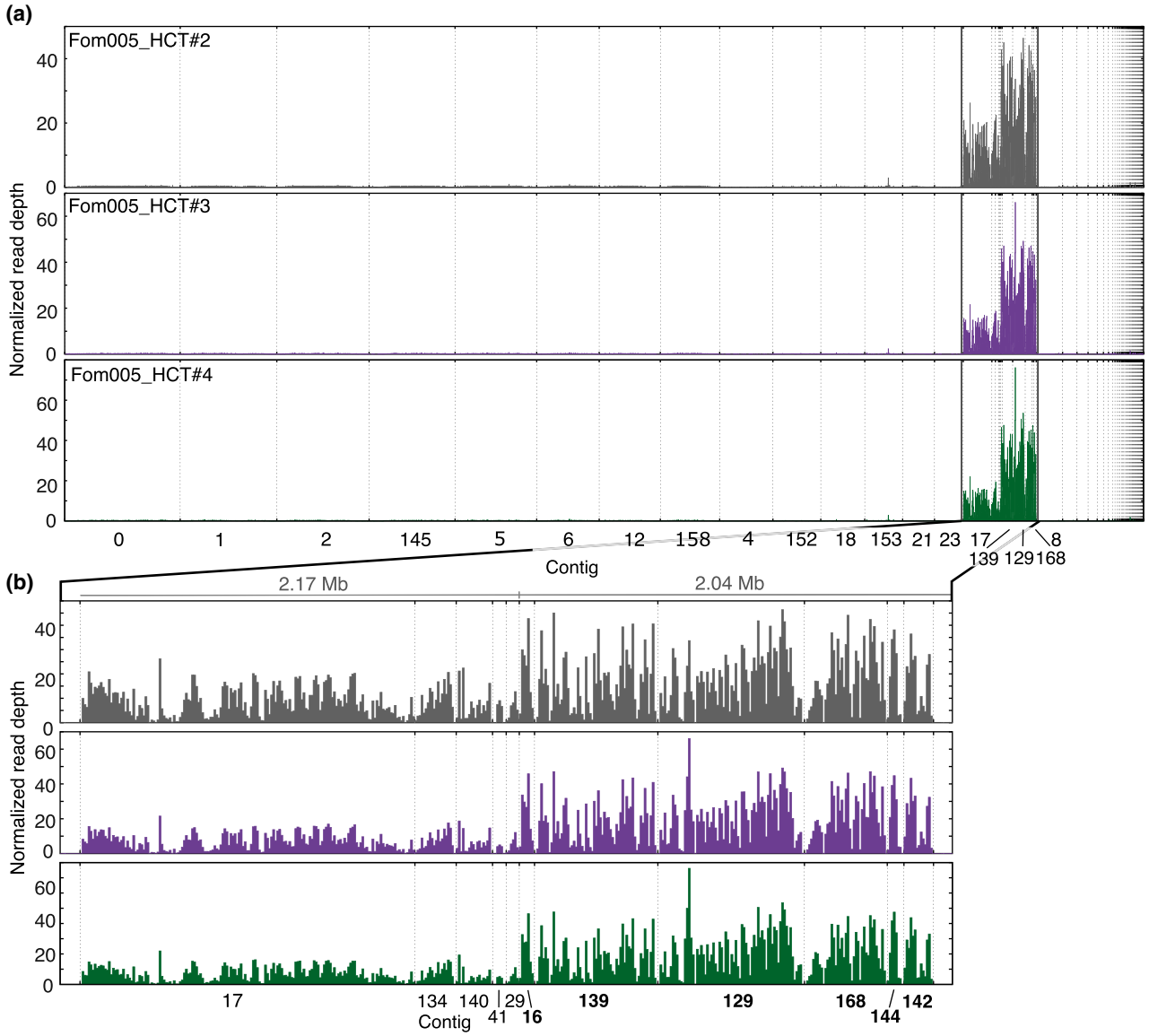

FIGURE 6 Normalized Illumina read mapping confirms horizontal chromosome transfer (HCT) from Fom005 to Fo47 and reveals duplications. Reads of HCT strains were first mapped to Fo47. Read-pairs that were not mapped to Fo47 were extracted and these unmapped reads were mapped to the single molecule real-time (SMRT) sequencing assembly of Fom005 (a). HCT strains Fom005_HCT#2 (grey line), #3 (purple line), and #4 (green line) all resulted from transfer of contigs 17 (1.6 Mb), 134 (c.0.2 Mb), 140 (c.0.2 Mb), 41 (c.0.1 Mb), 29 (c.0.1 Mb), 16 (c.0.1 Mb), 139 (c.0.6 Mb), 129 (c.0.7 Mb), 168 (c.0.4 Mb), 144 (c.0.1 Mb), and 142 (c.0.15 Mb) to Fo47. Read densities of contigs 16, 139, 129, 168, 144, and 142 were twice as high as those of contigs 17, 134, 140, 41, and 29 (b). Read densities were normalized by dividing by average read density

in Forc) that are recognized by cucumber and watermelon and elicit an immune response, or (b) virulence genes on chr^{RC} that are absent in Fom and that facilitate colonization and infection of cucumber and watermelon, or a combination of both (a) and (b). If the first hypothesis is true and the avirulence genes in Fom are present in nontransferred parts of the genomes, HCT strains of Fom should be able to cause disease on cucumber and watermelon plants. To test this, bioassays on cucumber and watermelon with Fom_HCT strains were performed. It turned out that none of the Fom_HCT strains could cause disease on cucumber or watermelon (Figure 7b,c). This excludes the possibility that avirulence genes are present in nontransferred parts of the Fom genomes. Among all transferred sequences of Fom001 and Fom005, only contigs 13 and 127 of Fom001 are syntenic with contigs 16, 139, 144, 129, and 168 of Fom005 (Figure S5). Therefore, genes that determine virulence on melon plants are most likely located on these shared regions. These shared regions between Fom001 HCT strains and Fom005 HCT strains are largely syntenic with chr^{RC} (Figure 3). However, around 300 kb of the repeat-rich region of chr^{RC} is absent in shared regions of transferred sequences of Fom001 and Fom005 (Figure 3) (van Dam *et al.*, 2017). This suggests that genes on the 300 kb region may determine virulence on melon and watermelon plants.

In conclusion, differences between transferred chromosomes of Fom001 or Fom005 and chr^{RC} determine differences in host range between Fom and Forc (Figure 8).

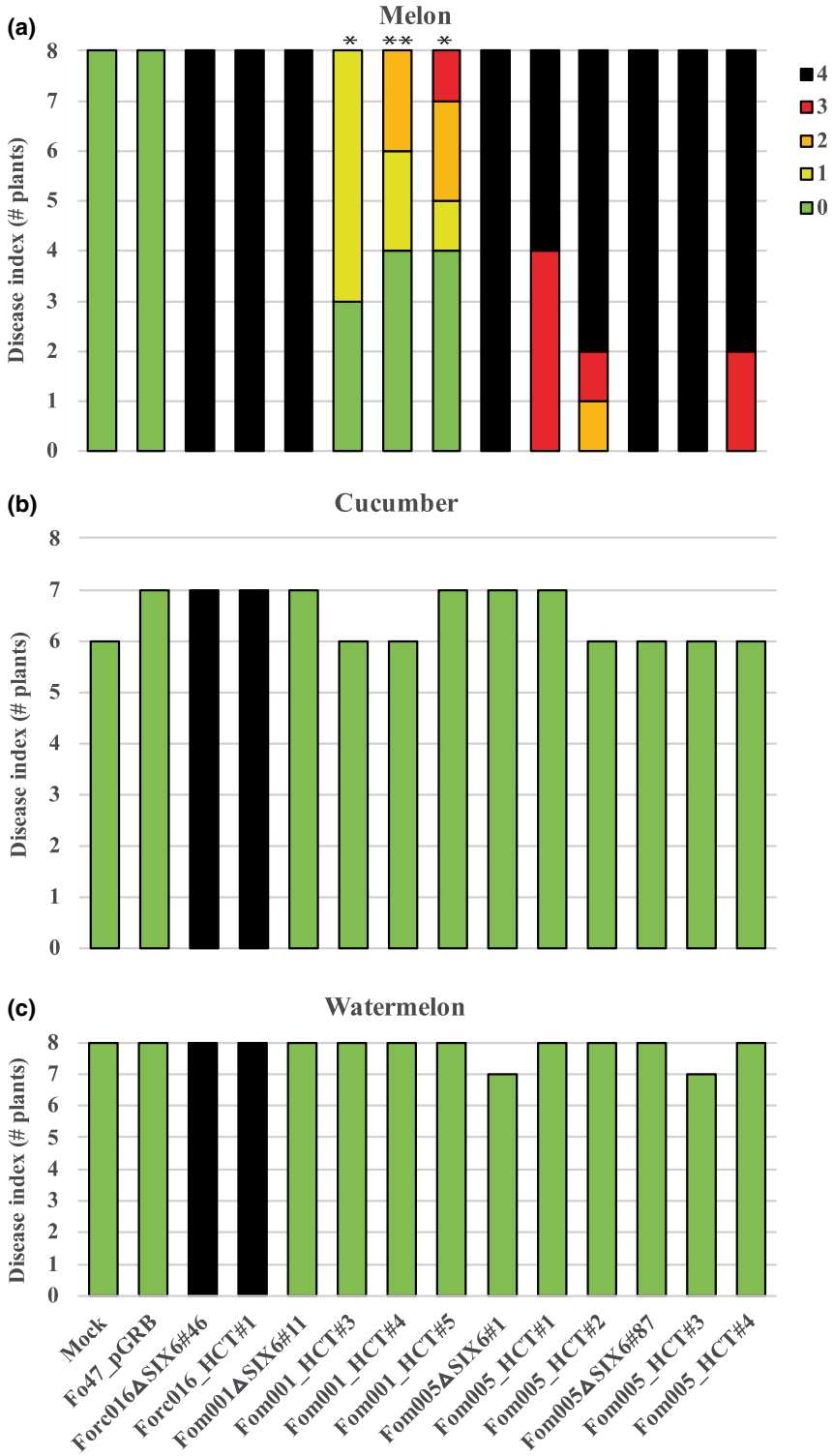

FIGURE 7 Transferred chromosomes of *Fusarium oxysporum* f. sp. *melonis* (Fom) determine virulence on melon and differences between transferred chromosomes of Fom and chr^{RC} determine differences in host range between Fom and *F. oxysporum* f. sp. *radicis-cucumerinum* (Forc). Six-day-old cucumber and 10-day-old melon and watermelon seedlings were inoculated with water (Mock) or strains (10^7 spores/ml) at 20 °C. The disease index of infected melon (a), cucumber (b), and watermelon (c) plants were scored 2 weeks after inoculation. All horizontal chromosome transfer (HCT) strains Fom001_HCT #3, #4, #5, Fom005_HCT #1, #2, #3, #4, and Forc016_HCT#1 caused disease on melon plants (a). While Fom001_HCT #3, #4, and #5 showed significantly reduced virulence compared to the donor strain Fom001ΔSIX6#11, Fom005_HCT #1, #2, #3, #4, and Forc016_HCT#1 showed no significantly reduced virulence compared to their respective donor strains. In contrast to Forc016_HCT#1, Fom-derived HCT strains did not cause disease on cucumber (b) or on watermelon (c). Kruskal–Wallis test was performed (*.05 < p < .01; **p < .01)

3 | DISCUSSION

We have found that both Forc and Fom are able to colonize xylem vessels of cucumber, melon, and watermelon plants, but colonization of cucumber and watermelon by Fom is limited and restricted to the roots. In addition, transferred chromosomes of two Fom strains can turn a nonpathogenic Fo strain into a melon-infecting strain. Lastly, we demonstrate that differences between transferred chromosomes of Fom and chr^{RC} determine differences in host range between Fom and Forc.

GFP-labelled cucurbit-infecting strain Forc016 and melon-infecting strain Fom005 were used to infect cucumber, melon, and watermelon and the colonization process was followed until 14 dpi. Even though Fom005_GFP did not cause disease symptoms on non-host cucumber and watermelon plants, it colonized root xylem vessels of all these plants by 3–4 dpi. Similarly, root xylem colonization

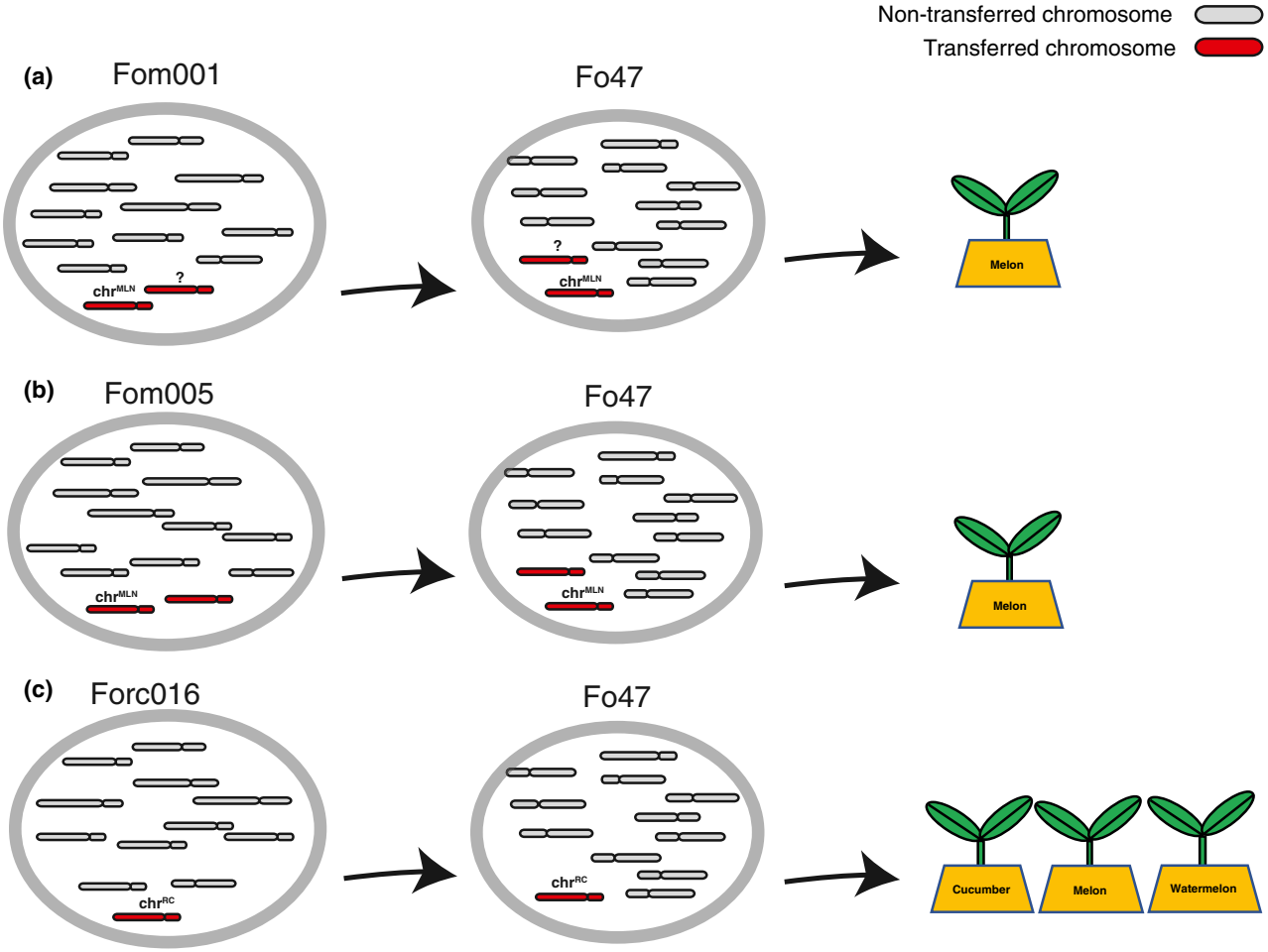

FIGURE 8 Schematic summary of chromosome transfers and their phenotypic effects. (a) For Fom001, the *SIX6*-containing chromosome, chr^{MLN}, which is around 2 Mb, together with (an)other chromosome(s) (labelled with "?") were transferred to Fo47, turning the recipient strain into a melon-infecting strain. (b) For Fom005, the *SIX6*-containing chromosome chr^{MLN} (c.2 Mb) was transferred to Fo47 together with another chromosome (c.2 Mb), thus the recipient strain is able to infect melon. (c) The *SIX6*-containing chromosome in Forc016 (chr^{RC}) was transferred to Fo47, and the recipient strain with chr^{RC} was able to infect cucumber, melon, and watermelon

of resistant host plants by Fo was observed in different plant species, including melon (Zvirin *et al.*, 2010), chickpea (Jimenez-Fernandez *et al.*, 2013), and common bean (Garces-Fiallos *et al.*, 2017). In most cases, however, colonization of xylem vessels of resistant plants by Fo was delayed compared to colonization of susceptible plants, and only the lower hypocotyl was colonized (Zvirin *et al.*, 2010) or only root xylem vessels were colonized (Li *et al.*, 2014). In other studies, colonization of xylem vessels of resistant plant roots by Fo was not observed (Cohen *et al.*, 2015; Upasani *et al.*, 2016). In the present study, colonization of the hypocotyl of cucumber and watermelon plants by Fom005_GFP was also not observed, suggesting strong defence responses of nonhosts cucumber and watermelon at the root–stem transition. Moreover, the biomass of Fom in cucumber roots was significantly less than of Forc (Figure 1), indicating that Fom005 growth was restricted in cucumber roots. Forc016_GFP-infected cucumber showed severe and fast root rot in 2 weeks, which possibly influenced the growth of new roots to compensate for the function of the original roots, resulting in completely destroyed roots and death of plants in less than 2 weeks.

Xylem colonization does not necessarily cause external disease symptoms. This raises the question of what allows plants to stay "symptom-free" on pathogen colonization. Our data suggest that fast-growing new roots (cucumber) may act as a strategy to compensate for the loss of function of infected roots. This may buy plants some time to employ other strategies to counteract infection more directly.

Effectors have been shown to contribute to virulence in various xylem-colonizing fungi (de Sain and Rep, 2015). While Fom causes root rot only in melon, Forc also causes severe root rot in cucumber and watermelon. This could be due to effector genes in Forc that allow cucumber and watermelon infection (van Dam *et al.*, 2017). Here, we show that Fom strains without *SIX6* do not show reduced virulence at tested conditions in melon plants. In contrast, *SIX6* has been shown to contribute to virulence in cucurbit-infecting strain Forc016 at 25 °C (van Dam *et al.*, 2017) as well as in a watermelon-infecting strain (Niu *et al.*, 2016). Deletion of *SIX6* in a tomato-infecting strain modestly compromised virulence in one study (Gawehns *et al.*, 2014), but in another study, deletion strains lacking a large part of the Fol

pathogenicity chromosome, including *SIX6*, *SIX9*, and *SIX11*, did not show significantly reduced virulence (Vlaardingerbroek *et al*., 2016b). *SIX6* of Forc016 does not contribute to virulence at 20 °C, indicating that the environment can influence the requirement of effectors. It could be that *SIX6* of Fom contributes to virulence in other conditions.

Effector genes in plant-pathogenic fungi are sometimes located on conditionally dispensable chromosomes, and some of these chromosomes can be transferred from one strain to another (Friesen *et al*., 2006; Akagi *et al*., 2009; Ma *et al*., 2010; van Dam *et al*., 2017). In Fo, HCT had been previously shown in Fol and Forc, and in both cases the recipient nonpathogenic strain became pathogenic to tomato and multiple cucurbits, respectively (Ma *et al*., 2010; van Dam *et al*., 2017). Here, we show that Fom strains can also engage in HCT. Both Fom001 and Fom005, which have different mating types, MAT1-1 (Fom005) and MAT1-2 (Fom001), could transfer one or more chromosomes to the same MAT1-2 (Fo47) strain. This is the first demonstration of HCT between the same mating type. Interestingly, in two different HCT experiments involving Fom001 and Fom005, besides transfer of the *SIX6*-containing chromosome, another chromosome was cotransferred (Figure 4). Cotransfer of a second nonselected chromosome has been previously observed (Ma *et al*., 2010). Transfer of a single chromosome from Fom001 or Fom005 was not found for any of the double-resistant colonies. This supports the hypothesis that HCT happens through nuclear fusion and selective chromosome loss or retainment rather than uptake of chromosomes by the recipient nucleus (Vlaardingerbroek *et al*., 2016a). How these chromosomes are selectively retained and replicated remains to be discovered. Following HCT, (a) large deletion(s) of the transferred chromosome was observed for HCT strain Fom001_HCT #5 (Figure 5). This strain showed no reduction in virulence compared to the other two Fom001_HCT strains, indicating that the lost region does not contribute to virulence. Transfer of genomic sequences of Fom001 was confirmed by whole-genome sequencing, but no extra bands were observed on the CHEF gel; it could be that the transferred regions or chromosomes acquired a size indistinguishable from a core chromosome in the CHEF gel.

With the development of next-generation sequencing technologies, prediction of putative (host-specific) virulence genes is relatively straightforward (Borah *et al*., 2018). However, functional verification of genes involved in the host range is still challenging. This can be due to the pathosystem under investigation and/or to the probability that multiple genes are collectively responsible for host specificity (Borah *et al*., 2018). In *Alternaria alternata*, host-specific toxin genes have been found in several pathotypes (Tsuge *et al*., 2013). Interestingly, host-selective toxin gene clusters in this fungus reside on single small chromosomes and these chromosomes can be combined by protoplast fusion, resulting in strains producing both toxins (Tsuge *et al*., 2013). Single chromosomes that determine the host range have also been identified through HCT (Ma *et al*., 2010; van Dam *et al*., 2017). In this way, genes involved in host specificity are narrowed down to single chromosomes. Here, too, we found chromosomes of Fom001 and Fom005 that determine virulence on melon plants through HCT. In addition, we demonstrated that differences between transferred chromosomes of Fom and chr^{RC} determine differences in host range between Fom and Forc. Because the shared regions of transferred chromosomes between Fom001 and Fom005 are largely syntenic with chr^{RC} (Figure 3), finding genes that are responsible for the differences in host range between Fom and Forc becomes possible.

Schulze-Lefert and Panstruga suggest that recognition of effectors by nonhosts (effector-triggered immunity) plays a predominant role in nonhost resistance in plant species closely related to a host species (Schulze-Lefert and Panstruga, 2011). *SIX1* is the only *SIX* gene homolog that is consistently present in Fom genomes but is not found in Forc (van Dam *et al*., 2017), suggesting that *SIX1* of Fom could be recognized by cucumber and watermelon. However, we found that Fom_HCT strains without *SIX1* still could not infect cucumber and watermelon plants, indicating that *SIX1* does not act as (the sole) avirulence gene in Fom for cucumber and watermelon. Of course, non-*SIX* homologs may also act as avirulence genes. Moreover, about 300 kb of sequence is present on chr^{RC} but absent on the shared regions of transferred chromosomes between Fom001 and Fom005. These sequences may contain virulence genes whose products can suppress immunity in cucumber and watermelon. Our future work will include finding genes on chr^{RC} or transferred chromosomes of Fom that determine the difference in host range between Fom and Forc.

In conclusion, we report here for the first time colonization differences between host and nonhost plants by Fo. Moreover, we show that largely homologous chromosomes determine virulence to their host plant(s) and are thus also responsible for differences in host range. This opens up the possibility of finding genes that determine differences in host range. Lastly, we demonstrate again that HCT plays an important role in environmental adaptation in Fo.

4 | EXPERIMENTAL PROCEDURES

4.1 | Cloning

Vector pRW2h_Pfem_GFP_Tsix1 was constructed by amplifying the GFP coding sequence from pPK2-HPH-GFP (Michielse and Rep, 2009) using primers FP740 (5′-AAAtctagaATGGTGAGCAAGGG CGAGGAG-3′, lowercase for *Xba*I restriction site) and FP7001 (5′-TTTagatctTTACTTGTA CAGCTCGTCC-3′, lower case for *Bgl*II restriction site) followed by *Xba*I-*Bgl*II digestion and inserting it into the *Xba*I-*Bgl*II site of pRW2h_Pfem_MCS_Tsix1 (van der Does *et al*., 2016). The hygromycin resistance cassette of pRW2h_Pfem_GFP_Tsix1 was replaced by the phleomycin resistance cassette of pRW1p_Pfem_MCS_Tsix1, which was modified from pRW1p (Houterman *et al*., 2008) using the same method as described by van der Does *et al*. (2016), resulting in pRW1p_Pfem_GFP_Tsix1.

To delete *SIX6* in Fom001 and Fom005, two *SIX6* deletion constructs were used. The first one, pPDh_SIX6, was generated previously (van Dam *et al*., 2017). The second *SIX6* deletion construct was

modified from pPK2-HPH-GFP (Michielse *et al*., 2009). First, around 1 kb *SIX6* flanks were amplified. One flank was amplified using primer pair FP7186 (5′-ATGATTACGAATTCTTAATTAAGATCCGAAGAGCTGGATCGTTTGAA-3′) and FP7187 (5′-CCATCAACTTGTCCTGTCACATCATCGAGCTCGGTACCCGGgatctt-3′), while another flank was amplified using primer pair FP7188 (5′-ctccactcgacctgcaggcatgcaCTATAAAGCCAATACGATTCGAA-3′) and FP7189 (5′-GCACGTGATCTAGGTTATTCTACtGGCACTGGCCGTCGTTTTACAAC-3′). These two flanks were introduced into pPK2-HPH-GFP using the HiFi cloning kit (New England Biolabs (UK) Ltd). Then, a *Pml*I fragment containing the herpes simplex virus thymidine kinase (*HSVtk*) gene under the control of the *Cochliobolus heterostrophus* glyceraldehyde-3-phosphate dehydrogenase (ChGPD) gene promoter and the *Neurospora crassa* β-tubulin gene terminator was inserted into the vector as a conditional negative selection marker against ectopic transformants (Khang *et al*., 2005).

4.2 | Generation of strains with green fluorescence

Agrobacterium-mediated *Fusarium* transformation was performed as described by (Takken *et al*., 2004). GFP-expressing T-DNA of pRW2h_Pfem_GFP_Tsix1 was integrated randomly into the genome of Forc016 ("33"; CBS141123) (Lievens *et al*., 2007; van Dam *et al*., 2016) and Fom005 (Fom0123) (Schmidt *et al*., 2013). Transformants Forc016_GFP (FP4379) and Fom005_GFP (FP4374) showing wild-type phenotype with green fluorescence were confirmed by fluorescence microscopy and selected for microscopic examination of fungal colonization.

4.3 | Microscopic examination of fungal colonization

Conidia were isolated from 5-day-old cultures grown in NO_3-medium (0.17% yeast nitrogen base, 3% sucrose, 100 mM KNO_3) by filtering through Miracloth (pore size 22–25 μm; Merck). Spores were centrifuged, resuspended in sterile milli-Q water, counted, and brought to a final concentration of 10^7 spores/ml. Roots of 6-day-old cucumber and 10-day-old melon and watermelon seedlings were carefully taken out of the soil and rinsed with tap water to wash away soil particles. Three plants for each treatment were inoculated with 10^7 spores/ml of Forc016_GFP, Fom005_GFP or water as control in a Petri dish (Figure S1). Colonization was examined at 1, 2, 3, 4, 6, and 14 dpi using the AMG Evos FL digital inverted microscope. GFP was excited with a 488-nm light (emission 525–550 nm BP filter). At 14 dpi, the hypocotyls of some plants were hand-sectioned and examined for the presence of fungal hyphae.

4.4 | Fungal biomass quantification using qPCR

The relative amount of fungal DNA in plant roots was quantified using qPCR. Nine-day-old cucumber and melon seedlings were inoculated with 10^7 spores/ml of Forc016, Fom001, or Fom005 (Schmidt *et al*., 2016) and roots from five plants for each treatment were randomly collected and rinsed with water 1 week after inoculation. DNA was isolated using GeneJET plant Genomic DNA purification Mini kit (Thermos Scientific). For detection and quantification of fungal DNA, primers FP8520 (5′-AGATTAGCGACACCCCTTGC-3′) and FP8521 (5′-TTTGGGCTCGTCTTCCACTG-3′) for amplifying the single-copy gene *SIX6* were used for Forc016, Fom001, and Fom005. Primers FP8434 (5′-CAGTGAAACAGGTGCAGGAA-3′) and FP8435 (5′-CTGGTGTAATGACCACGAGC-3′) were used for amplifying the *α-tubulin* gene of cucumber plants as an endogenous gene for normalization, while primers FP8512 (5′-GCGGTGCTTCTAGACAATGA-3′) and FP8513 (5′-CCTGAGATACAAGACGGTTGAG-3′) were used for amplifying the melon *α-tubulin* gene (Kong *et al*., 2014). qPCR was performed on a QuantStudio 3 system (ThermoFisher Scientific). A total volume of 10 μl of the reaction mixture included 2 μl of 5 × HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne), 5 pmol of each primer, 20 ng of template, and 1 μl of sterile milli-Q water. Three technical replicates were used for each sample to confirm the reproducibility of the results, and a negative control sample with sterile milli-Q water as template was included. Standard curves for all three primer pairs were generated and assessed. The PCR programme, including melting curve analysis, was set as follows: 15 min at 95 °C; 40 cycles of 15 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C; then 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. Data analysis was performed using Thermo Fisher Cloud RQ analysis tool combined with qbase.

4.5 | *SIX6* replacement

To replace *SIX6* in Fom001 and Fom005 with a hygromycin resistance cassette, *Agrobacterium*-mediated transformation was performed as previously described (Takken *et al*., 2004; van Dam *et al*., 2017). Following monosporing of hygromycin-resistant colonies, the transformants were grown in 96-well plates containing in each well 150 μl of potato dextrose broth (PDB) supplemented with hygromycin and 5 μM 5-fluoro-2-deoxyuridine (Alfa-Aesar) for preselection of *in locus* insertion of the construct through homologous recombination. Successful deletion of *SIX6* was confirmed by PCR using primers inside the T-DNA and outside the 1 kb flanking regions in the construct. Fom001ΔSIX6#11 and Fom005ΔSIX6#1 were generated using the construct of pPDh_SIX6, while Fom005ΔSIX6#87 was generated using pPK2-HPH-GFP_h_SIX6.

4.6 | Horizontal chromosome transfer

HCT experiments were performed by co-incubating Forc016ΔSIX6#46, Fom001ΔSIX6#11, Fom005ΔSIX6#1, and Fom005ΔSIX6#87 with Fo47pGRB (Vlaardingerbroek *et al*., 2016a), respectively. Conidia (10^5) from each pair of strains were mixed and co-incubated on potato dextrose agar (PDA) plates for 7 days at 25 °C. Microconidia were harvested from the co-incubation plate using 5 ml of sterile milli-Q water filtered

through sterile Miracloth and pipetted on a double-selective PDA plate containing 0.1 M Tris (pH 8) supplemented with 100 μg/ml hygromycin (Duchefa) and 100 μg/ml zeocin (InvivoGen). Double drug-resistant colonies were selected after 3 days and transferred to a new double-selective plate. Growing colonies were then monospored by spreading on a fresh plate supplemented with both antifungal drugs. After 2 days of growth, single-spore colonies were selected and transferred to fresh plates.

4.7 | Contour-clamped homogeneous electric field electrophoresis

Preparation of protoplasts and pulsed-field gel electrophoresis were performed as described previously (van Dam *et al.*, 2017). *Fusarium* strains were cultured in 100 ml NO_3 medium (0.17% yeast nitrogen base, 100 mM KNO_3, and 3% sucrose) for 5 days at 25 °C. Then, conidia were collected by filtering through a double layer of Miracloth and the concentration of spores was measured. Conidia (5×10^8) were transferred to 40 ml PDB (BD Biosciences). After approximately 16 hr of growth at 25 °C, germinated spores were resuspended in 10 ml of $MgSO_4$ solution (1.2 M $MgSO_4$, 50 mM sodium citrate, pH 5.8) supplemented with 50 mg/ml Glucanex (Sigma) + 5 mg/ml driselase (Sigma) and incubated for approximately 17 hr at 30 °C in a shaking water bath (65 rpm). The protoplasts were filtered through a double layer of Miracloth, collected by centrifugation and, cast in InCert agarose (Lonza) at a concentration of 2×10^8 protoplasts per millilitre. Plugs were treated with 2 mg/ml pronase E at 50 °C. Chromosomes were separated by pulsed-field electrophoresis for 260 hr in 1% Seakem Gold agarose (Lonza) at 1.5 V/cm in a CHEF-DRII system (Biorad) in 0.5 × Tris-borate-EDTA (TBE) buffer at 4 °C, with switch times between 1,200 and 4,800 s. The gels were stained with 1 μg/ml ethidium bromide in 0.5 × TBE.

4.8 | DNA isolation, genome sequencing, and assembly

DNA isolation was performed on freeze-dried mycelium ground in liquid nitrogen as starting material, using multiple rounds of phenol–chloroform extraction and precipitation, as well as the Purelink plant total DNA purification kit (Invitrogen).

SMRT sequencing was performed at Keygene N.V. (Wageningen, Netherlands). PacBio libraries were prepared and size-selected at c.20 kb using Blue Pippin prep. Sequencing of five SMRT cells was performed using the P6-C4 polymerase-chemistry combination, ≥4 hr movie time, stage start. De novo assembly was performed with the Hierarchical Genome Assembly Process v. 3 (HGAP.3, Pacific Biosciences) within the SMRT Portal environment (v. 1.87.139483). Default values were kept and the expected genome size was set to 60 Mb.

Illumina sequencing (150 bp paired-end, insert size c.500 bp) of HCT strains was performed on a HiSeq 2,500 machine at the Hartwig Medical Foundation (Amsterdam, Netherlands) at c.100 × coverage, resulting in 5.0–5.6 Mb of sequence data per sample.

Raw reads were trimmed to remove low-quality bases and adapter sequences using fastq-mcf v. 1.04.807 (−q 20). PCR duplicates were also removed using PicardTools MarkDuplicates v. 2.7.1 with standard settings. To confirm HCT, trimmed reads were first mapped against the Fo47 genome assembly with Bowtie2 v. 2.2.5 (DNAseq). Unmapped reads were selected from the bamfile that resulted from mapping trimmed reads to the assembly of Fo47 with SAMtools view −f 4 −F 264 (both mates unmapped), SAMtools view −f 8 −F 260 (mate 1 mapped, other mate unmapped), and SAMtools view −f 12 −F 256 (mate 2 mapped, other mate unmapped). The three resulting bamfiles were sorted with SAMtools sort −n, merged with SAMtools merge and converted to fastq format with bedtools bamtofastq. These reads were then mapped to the SMRT assembly of Fom001 or Fom005, respectively. To establish whether core chromosomes were transferred, trimmed reads were directly mapped to SMRT assembly of Fom001 or Fom005, and only reads that mapped once with 100% coverage and 100% identity were selected (with SAMtools view −q 42) when calculating read densities. For visualization of the reads counts in 10 kb nonoverlapping sliding windows, SAMtools bedcov was used. SAMtools v. 1.8 was used in all above-mentioned cases.

Whole-genome or chromosome alignments were performed using nucmer (with −maxmatch or otherwise default settings) from the MUMmer v. 3.23 package (Delcher *et al.*, 2002).

4.9 | Virulence assay

Virulence assays were performed as described previously (van Dam *et al.*, 2017). Briefly, spores at 10^6/ml (*SIX6* deletion assay) or 10^7/ml (chromosome transfer) were used to inoculate seedlings of cucumber (around 6–7 days old), melon (9–10 days old), or watermelon (9–10 days old). For each treatment, six to eight seedlings were inoculated and grown at 25 °C (*SIX6* deletion assay) or 20 °C (HCT assay) in a greenhouse. The following plant cultivars were used: *Cucumis sativus* 'Paraiso', *Cucumis melo* 'Cha-T', and *Citrullus lanatus* 'Black Diamond'. Two weeks after inoculation, disease symptoms were scored using a disease index ranging from 0 to 4 (0, no symptoms; 1, slight root rot symptoms, only at tip of main root; 2, root rot symptoms and stem lesions visible aboveground; 3, extensive rot of the entire root system, often with a large lesion extending above the cotyledons; 4, plant either dead or very small and wilted).

ACKNOWLEDGEMENTS

We are grateful to Lee James Conneely for help with HCT experiments, Lotje van der Does for providing pRW2h_Pfem_MCS_Tsix1 construct, Maria Constantin for critical reading of the paper, Harold Lemereis and Ludek Tikovsky for plant care, ENZA seeds for kindly providing cucumber and melon seeds, and Rijk Zwaan for kindly providing watermelon seeds. J.L. was financially supported by the China Scholarship Council program (file number: 201504910768). L.F. was

financially supported by the NWO Talent Scheme Veni (grant number: 016.veni.181.090).

CONFLICT OF INTEREST

No conflict of interest is declared.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ORCID

Jiming Li https://orcid.org/0000-0001-7443-7748
Martijn Rep https://orcid.org/0000-0003-3608-6283

REFERENCES

Akagi, Y., Akamatsu, H., Otani, H. and Kodama, M. (2009) Horizontal chromosome transfer, a mechanism for the evolution and differentiation of a plant-pathogenic fungus. *Eukaryotic Cell*, 8, 1732–1738.

Bani, M., Pérez-De-Luque, A., Rubiales, D. and Rispail, N. (2018) Physical and chemical barriers in root tissues contribute to quantitative resistance to *Fusarium oxysporum* f. sp *pisi* in pea. *Frontiers in Plant Science*, 9, 199.

Borah, N., Albarouki, E. and Schirawski, J. (2018) Comparative methods for molecular determination of host-specificity factors in plant-pathogenic fungi. *International Journal of Molecular Sciences*, 19(3), 863.

Bubici, G., Kaushal, M., Prigigallo, M.I., Cabanas, C.G.L. and Mercado-Blanco, J. (2019) Biological control agents against fusarium wilt of banana. *Frontiers in Microbiology*, 10, 616.

Cohen, R., Orgil, G., Burger, Y., Saar, U., Elkabetz, M., Tadmor, Y. *et al.* (2015) Differences in the responses of melon accessions to fusarium root and stem rot and their colonization by *Fusarium oxysporum* f. sp *radicis-cucumerinum*. *Plant Pathology*, 64, 655–663.

Croll, D. and McDonald, B.A. (2012) The accessory genome as a cradle for adaptive evolution in pathogens. *PLoS Pathogens*, 8(4), e1002608.

van Dam, P., Fokkens, L., Schmidt, S.M., Linmans, J.H., Kistler, H.C., Ma, L.J. *et al.* (2016) Effector profiles distinguish formae speciales of *Fusarium oxysporum*. *Environmental Microbiology*, 18, 4087–4102.

van Dam, P., Fokkens, L., Ayukawa, Y., van der Gragt, M., Ter Horst, A., Brankovics, B. *et al.* (2017) A mobile pathogenicity chromosome in *Fusarium oxysporum* for infection of multiple cucurbit species. *Scientific Reports*, 7, 9042.

Delcher, A.L., Phillippy, A., Carlton, J. and Salzberg, S.L. (2002) Fast algorithms for large-scale genome alignment and comparison. *Nucleic Acids Research*, 30, 2478–2483.

Di Pietro, A., Madrid, M.P., Caracuel, Z., Delgado-Jarana, J. and Roncero, M.I.G. (2003) *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. *Molecular Plant Pathology*, 4, 315–325.

van der Does, H.C., Fokkens, L., Yang, A., Schmidt, S.M., Langereis, L., Lukasiewicz, J.M. *et al.* (2016) Transcription factors encoded on core and accessory chromosomes of *Fusarium oxysporum* induce expression of effector genes. *PLoS Genetics*, 12, e1006401.

Friesen, T.L., Stukenbrock, E.H., Liu, Z., Meinhardt, S., Ling, H., Faris, J.D. *et al.* (2006) Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature Genetics*, 38, 953–956.

Garces-Fiallos, F.R., de Borba, M.C., Schmidt, E.C., Bouzon, Z.L. and Stadnik, M.J. (2017) Delayed upward colonization of xylem vessels is associated with resistance of common bean to *Fusarium oxysporum* f. sp. *phaseoli*. *European Journal of Plant Pathology*, 149, 477–489.

Gawehns, F., Houterman, P.M., Ichou, F.A., Michielse, C.B., Hijdra, M., Cornelissen, B.J.C. *et al.* (2014) The *Fusarium oxysporum* effector Six6 contributes to virulence and suppresses *I-2*-mediated cell death. *Molecular Plant-Microbe Interactions*, 27, 336–348.

Houterman, P.M., Cornelissen, B.J. and Rep, M. (2008) Suppression of plant resistance gene-based immunity by a fungal effector. *PLoS Pathogens*, 4, e1000061.

Houterman, P.M., Ma, L., van Ooijen, G., de Vroomen, M.J., Cornelissen, B.J., Takken, F.L. *et al.* (2009) The effector protein Avr2 of the xylem-colonizing fungus *Fusarium oxysporum* activates the tomato resistance protein I-2 intracellularly. *The Plant Journal*, 58, 970–978.

de Jonge, R., Bolton, M.D., Kombrink, A., van den Berg, G.C.M., Yadeta, K.A. and Thomma, B. (2013) Extensive chromosomal reshuffling drives evolution of virulence in an asexual pathogen. *Genome Research*, 23, 1271–1282.

de Jonge, R., van Esse, H.P., Maruthachalam, K., Bolton, M.D., Santhanam, P., Saber, M.K. *et al.* (2012) Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 5110–5115.

Jimenez-Fernandez, D., Landa, B.B., Kang, S., Jimenez-Diaz, R.M. and Navas-Cortes, J.A. (2013) Quantitative and microscopic assessment of compatible and incompatible interactions between chickpea cultivars and *Fusarium oxysporum* f. sp. *ciceris* races. *PLoS ONE*, 8, e61360.

Khang, C.H., Park, S.Y., Lee, Y.H. and Kang, S.C. (2005) A dual selection based, targeted gene replacement tool for *Magnaporthe grisea* and *Fusarium oxysporum*. *Fungal Genetics and Biology*, 42, 483–492.

Kistler, H.C. (1997) Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum*. *Phytopathology*, 87, 474–479.

Kong, Q., Yuan, J., Niu, P., Xie, J., Jiang, W., Huang, Y. *et al.* (2014) Screening suitable reference genes for normalization in reverse transcription quantitative real-time PCR analysis in melon. *PLoS ONE*, 9, e87197.

Li, E., Wang, G., Yang, Y., Xiao, J., Mao, Z. and Xie, B. (2014) Microscopic analysis of the compatible and incompatible interactions between *Fusarium oxysporum* f. sp. *conglutinans* and cabbage. *European Journal of Plant Pathology*, 141, 597–609.

Lievens, B., Claes, L., Vakalounakis, D.J., Vanachter, A.C.R.C. and Thomma, B.P.H.J. (2007) A robust identification and detection assay to discriminate the cucumber pathogens *Fusarium oxysporum* f. sp *cucumerinum* and f. sp *radicis-cucumerinum*. *Environmental Microbiology*, 9, 2145–2161.

Ma, L., Houterman, P.M., Gawehns, F., Cao, L., Sillo, F., Richter, H. *et al.* (2015) The *AVR2-SIX5* gene pair is required to activate *I-2*-mediated immunity in tomato. *New Phytologist*, 208, 507–518.

Ma, L.J., van der Does, H.C., Borkovich, K.A., Coleman, J.J., Daboussi, M.J., Di Pietro, A. *et al.* (2010) Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature*, 464, 367–373.

Mehrabi, R., Bahkali, A.H., Abd-Elsalam, K.A., Moslem, M., Ben M'Barek, S., Gohari, A.M. *et al.* (2011) Horizontal gene and chromosome transfer in plant pathogenic fungi affecting host range. *FEMS Microbiology Reviews*, 35, 542–554.

Michielse, C.B. and Rep, M. (2009) Pathogen profile update: *Fusarium oxysporum*. *Molecular Plant Pathology*, 10, 311–324.

Michielse, C.B., van Wijk, R., Reijnen, L., Cornelissen, B.J.C. and Rep, M. (2009) Insight into the molecular requirements for pathogenicity of *Fusarium oxysporum* f. sp. *lycopersici* through large-scale insertional mutagenesis. *Genome Biology*, 10, R4.

Niño-Sánchez, J., Tello, V., Casado-Del Castillo, V., Thon, M.R., Benito, E.P. and Diaz-Mínguez, J.M. (2015) Gene expression patterns and dynamics of the colonization of common bean (*Phaseolus vulgaris* L.) by highly virulent and weakly virulent strains of *Fusarium oxysporum*. *Frontiers in Microbiology*, 6, 234.

Niu, X.W., Zhao, X.Q., Ling, K.S., Levi, A., Sun, Y.Y. and Fan, M. (2016) The *FonSIX6* gene acts as an avirulence effector in the *Fusarium oxysporum* f. sp. *niveum*—watermelon pathosystem. *Scientific Reports*, 6, 28146.

Perez-Nadales, E., Nogueira, M.F.A., Baldin, C., Castanheira, S., El Ghalid, M., Grund, E. *et al.* (2014) Fungal model systems and the elucidation

of pathogenicity determinants. *Fungal Genetics and Biology*, 70, 42–67.
Rep, M., van der Does, H.C., Meijer, M., van Wijk, R., Houterman, P.M., Dekker, H.L. *et al.* (2004) A small, cysteine-rich protein secreted by *Fusarium oxysporum* during colonization of xylem vessels is required for *I-3*-mediated resistance in tomato. *Molecular Microbiology*, 53, 1373–1383.
de Sain, M. and Rep, M. (2015) The role of pathogen-secreted proteins in fungal vascular wilt diseases. *International Journal of Molecular Sciences*, 16, 23970–23993.
Schmidt, S.M., Houterman, P.M., Schreiver, I., Ma, L., Amyotte, S., Chellappan, B. *et al.* (2013) MITEs in the promoters of effector genes allow prediction of novel virulence genes in *Fusarium oxysporum*. *BMC Genomics*, 14, 119.
Schmidt, S.M., Lukasiewicz, J., Farrer, R., van Dam, P., Bertoldo, C. and Rep, M. (2016) Comparative genomics of *Fusarium oxysporum* f. sp. *melonis* reveals the secreted protein recognized by the *Fom-2* resistance gene in melon. *New Phytologist*, 209, 307–318.
Schulze-Lefert, P. and Panstruga, R. (2011) A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. *Trends in Plant Science*, 16, 117–125.
Takken, F.L.W., van Wijk, R., Michielse, C.B., Houterman, P.M., Ram, A.F.J. and Cornelissen, B.J.C. (2004) A one-step method to convert vectors into binary vectors suited for *Agrobacterium*-mediated transformation. *Current Genetics*, 45, 242–248.
Tsuge, T., Harimoto, Y., Akimitsu, K., Ohtani, K., Kodama, M., Akagi, Y. *et al.* (2013) Host-selective toxins produced by the plant pathogenic fungus *Alternaria alternata*. *FEMS Microbiology Reviews*, 37, 44–66.
Upasani, M.L., Gurjar, G.S., Kadoo, N.Y. and Gupta, V.S. (2016) Dynamics of colonization and expression of pathogenicity related genes in *Fusarium oxysporum* f. sp. *ciceri* during chickpea vascular wilt disease progression. *PLoS ONE*, 11, e0156490.
Vlaardingerbroek, I., Beerens, B., Rose, L., Fokkens, L., Cornelissen, B.J. and Rep, M. (2016a) Exchange of core chromosomes and horizontal transfer of lineage-specific chromosomes in *Fusarium oxysporum*. *Environmental Microbiology*, 18, 3702–3713.
Vlaardingerbroek, I., Beerens, B., Schmidt, S.M., Cornelissen, B.J. and Rep, M. (2016b) Dispensable chromosomes in *Fusarium oxysporum* f. sp. *lycopersici*. *Molecular Plant Pathology*, 17, 1455–1466.
Zhang, M., Xu, J.H., Liu, G., Yao, X.F., Li, P.F. and Yang, X.P. (2015) Characterization of the watermelon seedling infection process by *Fusarium oxysporum* f. sp *niveum*. *Plant Pathology*, 64, 1076–1084.
Zvirin, T., Herman, R., Brotman, Y., Denisov, Y., Belausov, E., Freeman, S. *et al.* (2010) Differential colonization and defence responses of resistant and susceptible melon lines infected by *Fusarium oxysporum* race 1.2. *Plant Pathology*, 59, 576–585.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

FIGURE S1 Phenotypes of infected cucumber and melon plants in soil at 7 days post-inoculation. Nine-day-old cucumber and melon seedlings were treated with water (Mock), Forc016 (10^7 spores/ml), Fom001 (10^7 spores/ml), or Fom005 (10^7 spores/ml)

FIGURE S2 Phenotypes of infected cucumber, melon, and watermelon plants in Petri dishes at 14 days post-inoculation. Six-day-old cucumber and 10-day-old melon and watermelon seedlings were treated with water (Mock), Forc016_GFP (10^7 spores/ml), or Fom005_GFP (10^7 spores/ml) in Petri dishes for 14 days. Forc016_GFP causes severe wilting, and root and stem rot on cucumber, melon, and watermelon plants, while Fom005_GFP causes disease on melon, but not on cucumber or watermelon. All infected roots were dark brown compared to mock-treated roots. Fom005_GFP-infected cucumber plants produced many new roots, comparable to mock-treated cucumber plants. All mock-treated plants were healthy

FIGURE S3 Fom005 is not able to colonize the hypocotyl of cucumber or watermelon plants. The hypocotyls of infected or mock-treated plants at 14 days post-inoculation in Petri dishes were hand-sectioned and examined for the presence of fungal hyphae inside plants using an AMG Evos FL digital inverted microscope. Forc016_GFP was found in the cross-section of the hypocotyl of both cucumber (bright green dots indicated with white arrows) and watermelon (not shown). It was not possible to visualize Forc016_GFP in the hypocotyl of melon because of rotted stems. Fom005_GFP was present in the cross-section of the melon hypocotyl (bright green dots indicated with a white arrow), but was not found in the cross-section of hypocotyls of cucumber or watermelon (not shown)

FIGURE S4 *SIX6* does not contribute to the virulence of Fom001 and Fom005. Disease index (DI) (a) and fresh weight (b) of infected melon plants were scored 2 weeks after inoculation. When 10-day-old melon seedlings were inoculated with 10^6 spores/ml at 25 °C, the *SIX6* knockout strains Fom001ΔSIX6#11, #34, and Fom005ΔSIX6#1, #17 showed similar disease index and fresh weight as wild-type strains Fom001 and Fom005, respectively. As controls, disease symptoms of two ectopic transformants (T-DNA was randomly inserted in the genome) from each background were assessed, and these also showed no change in virulence compared to the wild-type strains. The Kruskal–Wallis test was performed on disease index. One-way analysis of variance was performed on fresh weight

FIGURE S5 Syntenic regions between transferred contigs of Fom001 and Fom005. Among all transferred contigs of Fom001 and Fom005, only contigs 127 and 13 of Fom001 are syntenic with contigs 139, 144, 129, 168, and 16 (nucmer alignment with manual adjustment). Alignments are colour-coded according to their similarity

FIGURE S6 Stringent selection of mapped Illumina reads of horizontal chromosome transfer (HCT) strains and Fom005 to the single molecule real-time (SMRT) sequencing assembly of Fom001 or Fom005 shows the absence of core chromosome transfer. (a) Reads of HCT strains Fom001_HCT #3, #4, and #5 were mapped to the SMRT assembly of Fom001 donor strain. (b) Reads of Fom001_HCT #3 (blue line), #4 (green line), and Fom001_HCT #5 (red line) mapped more abundantly to Fom001 contigs 124, 127, 3, and 13 than to the rest of the assembly. (c) Reads of HCT strains Fom005_HCT #2, #3, and #4 were mapped to the SMRT assembly of Fom005 donor strain. (d) Reads of Fom005_HCT#2 (blue line), #3 (green line), and #4 (red line) mapped more abundantly to Fom005 contigs 129, 139, 168, 142, 144, and 16 than to the rest of the assembly. (e) Reads of Fom005_HCT#2 (blue line), #3 (green line), and #4 (red line) and Fom005 (dark blue line) were mapped to the SMRT assembly of the Fom005 donor strain. Fom005 HCT strains showed clear read density drops at subtelomere regions, but no clear drop was observed

for Fom005 (dark blue line). Only reads that mapped once with 100% coverage and 100% identity were selected, to differentiate between HCT strains and the donor strain Fom001 or Fom005 based on single nucleotide polymorphisms

TABLE S1 Assembly statistics of assemblies of Fom005 and Fo47 that were generated for this study, and of Fom001 (van Dam *et al*., 2017)

How to cite this article: Li J, Fokkens L, van Dam P, Rep M. Related mobile pathogenicity chromosomes in *Fusarium oxysporum* determine host range on cucurbits. *Molecular Plant Pathology*. 2020;21:761–776. https://doi.org/10.1111/mpp.12927