Extensive chromosomal reshuffling drives evolution of virulence in an asexual pathogen

Ronnie de Jonge,[1,6] Melvin D. Bolton,[2,4] Anja Kombrink,[1,4] Grardy C.M. van den Berg,[1,4] Koste A. Yadeta,[1,5] and Bart P.H.J. Thomma[1,3,7]

[1]*Laboratory of Phytopathology, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands;* [2]*United States Department of Agriculture, Agricultural Research Service, Northern Crop Science Laboratory, Fargo, North Dakota 58102-2765, USA;* [3]*Centre for BioSystems Genomics, 6700 AB Wageningen, The Netherlands*

Sexual recombination drives genetic diversity in eukaryotic genomes and fosters adaptation to novel environmental challenges. Although strictly asexual microorganisms are often considered as evolutionary dead ends, they comprise many devastating plant pathogens. Presently, it remains unknown how such asexual pathogens generate the genetic variation that is required for quick adaptation and evolution in the arms race with their hosts. Here, we show that extensive chromosomal rearrangements in the strictly asexual plant pathogenic fungus *Verticillium dahliae* establish highly dynamic lineage-specific (LS) genomic regions that act as a source for genetic variation to mediate aggressiveness. We show that such LS regions are greatly enriched for in planta-expressed effector genes encoding secreted proteins that enable host colonization. The LS regions occur at the flanks of chromosomal breakpoints and are enriched for retrotransposons and other repetitive sequence elements. Our results suggest that asexual pathogens may evolve by prompting chromosomal rearrangements, enabling rapid development of novel effector genes. Likely, chromosomal reshuffling can act as a general mechanism for adaptation in asexually propagating organisms.

[Supplemental material is available for this article.]

Sexual reproduction is thought to enable responses to environmental challenges in nature and fosters evolution of pathogen genomes (Williams 1975; Maynard Smith 1978; Colegrave 2002; Goddard et al. 2005; Heitman 2006; de Visser and Elena 2007). Consequently, pathogen populations that undergo regular sexual reproduction are thought to pose a great risk to agriculture because they can recombine alleles that contribute to virulence in the face of dynamic environmental conditions (McDonald and Linde 2002). However, sexual reproduction comes at a cost because two compatible individuals need to locate each other to generate offspring, and fitness decreases due to break up of coadapted combinations of interacting alleles may occur (Agrawal 2006; Heitman 2006; Heitman et al. 2007; de Visser and Elena 2007). Sexual compatibility between individuals depends on so-called mating types in fungi. In heterothallic fungi, mating is restricted to genetically different individuals of opposite mating types, whereas in homothallic fungi, mating and sexual reproduction can occur between all individuals, including itself (Billiard et al. 2012). Although most fungi are able to undergo asexual and sexual reproduction, ~20% of all fungal phyla reproduce strictly asexually (Heitman et al. 2007). Such strictly asexual organisms are thought to be less flexible than sexual ones, relying solely on random mutations to adapt to changing environments. Asexual organisms are often considered as evolutionary dead ends (Burt 2000; McDonald and Linde 2002). This is mainly due to the absence of meiotic recombination resulting in increased accumulation of deleterious mutations, an effect known as Muller's ratchet (Felsenstein 1974), and a decreased ability to adapt to changing environmental conditions by generation of novel genetic combinations (Heitman 2006). Especially for pathogenic species that are continuously involved in an arms race with their hosts that try to ward off invading pathogens, quick adaptation in order to coevolve with the host immune system is essential for evolutionary success (Raffaele and Kamoun 2012). Nonetheless, many destructive plant pathogenic fungi are known as strictly asexually reproducing organisms, such as the vascular wilt fungus *Verticillium dahliae* (Fradin and Thomma 2006).

V. dahliae is a soil-borne broad host-range plant pathogen that invades the water-conducting xylem vessels of susceptible plant species to cause vascular wilt disease (Fradin and Thomma 2006; Klosterman et al. 2009). Hundreds of dicotyledonous plant species can be infected by *V. dahliae*, including many economically important crops such as lettuce, cotton, and tomato (Bolek et al. 2005; Fradin and Thomma 2006; Klosterman et al. 2009; Atallah et al. 2010). Although two mating types have been found in *V. dahliae*, it is predicted that propagation is solely asexual (clonal) in most if not all populations, as a sexual cycle has never been observed and the ratio between both mating types is greatly skewed toward one of the two (Usami et al. 2008, 2009; Atallah et al. 2010; Inderbitzin et al. 2011). Only in a few plant species has monogenic resistance toward Verticillium wilt been described. These include tomato, for which a *Verticillium* resistance locus (named *Ve*) has been identified that mediates resistance against race 1 strains of *V. dahliae* and *V. albo-atrum*, and that thus become avirulent (unable to cause disease). Strains that are not contained by this *Ve* locus remain virulent (able to cause disease) and are assigned to race 2 (Schaible et al. 1951; Kawchuk et al. 2001; Fradin et al. 2009). The resistance that is established by the *Ve* locus is

[4]These authors contributed equally to this work.
Present addresses: [5]Department of Plant Pathology, University of California, Davis, CA 95616, USA; [6]VIB Department of Plant Systems Biology, Ghent University, Bioinformatics and Evolutionary Genomics Division, Technologiepark 927, B-9052 Ghent, Belgium.
[7]Corresponding author
E-mail bart.thomma@wur.nl
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mediated by the *Ve1* gene that encodes a receptor-like protein-type cell surface receptor (Fradin et al. 2009) that activates plant immunity upon perception of a corresponding pathogen-derived ligand. Recently, through comparative genomics the race 1-specific effector Ave1 (for Avirulence on Ve1 tomato) was identified that activates Ve1-mediated resistance in tomato (de Jonge et al. 2012). Remarkably, the Ave1 effector contributes to pathogen aggressiveness on tomato plants that lack *Ve1*, demonstrating that Ave1 acts as a virulence factor of *V. dahliae* (de Jonge et al. 2012). Race 2 strains lack the *Ave1* gene, and are consequently less aggressive on tomato plants that lack *Ve1* when compared with race 1 strains (Amen and Shoemaker 1985; Paternotte and van Kesteren 1993; de Jonge et al. 2012).

In this study, we compared the genomes of 11 recently sequenced *V. dahliae* strains (Klosterman et al. 2011; de Jonge et al. 2012) that have been isolated from various geographical locations and hosts, and that are all, except for one, pathogenic on tomato. By assessing genetic diversity within this population we aimed to study the impact of asexual reproduction on the evolution of host adaptation and virulence in this plant pathogenic fungus.

Results

Identification of the core genome reveals extensive chromosomal rearrangements

With comparative genomics, we examined genetic diversity in a population of nine recently sequenced tomato pathogenic strains and one nonpathogenic strain of the asexual fungus *Verticillium dahliae* (Supplemental Table 1; de Jonge et al. 2012) together with the genome of *V. dahliae* strain VdLs17 as a reference (Klosterman et al. 2011). Read coverage mapping in 1-kb windows of all sequenced *V. dahliae* strains over the VdLs17 reference genome revealed a "core genome" that is shared by all strains, encompassing ~29 Mb of sequence containing 9471 genes. The amount of single nucleotide polymorphisms (SNPs) within the core genome ranged from 5445 (JR2; ~99.98% identity) to 163,602 (St.100; ~99.5% identity) SNPs per strain (Table 1), collectively amounting to 236,785 nonredundant polymorphic sites. Of these, 78,342 (32.9%) occurred in protein-coding regions, of which 55% were synonymous, not affecting the protein sequence, and 45% were nonsynonymous (Table 1). To determine selection strength, the ratio of nonsynonymous (d_N) substitutions per nonsynonymous site (K_a) to the number of synonymous substitutions (d_S) per synonymous site (K_s) was calculated within the coding regions for each of the 9471 core genes (Stukenbrock et al. 2010). A surprisingly low number of 29 genes was found to be under positive selection ($K_a/K_s > 1$; $P < 0.01$). About half of these (13) encode proteins with a known function, including cellulose binding and regulation of transcription, whereas the other half encodes proteins of unknown function, of which only three encode secreted proteins that are candidate effectors (Supplemental Table 2; de Jonge et al. 2011).

To infer evolutionary relations within the population, all 236,785 SNP positions were used to construct a phylogenetic tree (Fig. 1). Despite the high degree of conservation between the strains that were included in the population (maximum nucleotide divergence within the population is only 0.5%), strain JR2 formed a clearly separate cluster with the VdLs17 reference strain, differing only by one SNP in ~6000 bp (0.02%). Despite the high degree of identity (99.98%), the two strains show differential aggressiveness on tomato. Strain JR2 belongs to race 1 and shows a high degree of aggressiveness on tomato lacking *Ve1,* while VdLs17 belongs to race 2 and displays a relatively mild aggressiveness on tomato lacking *Ve1*. These two strains were analyzed in further detail. To improve the de novo JR2 genome assembly, mate-pair sequencing of a 5-kb insert library was performed, leading to a drastically improved contig N50 from 59.4 kb to 1.0 Mb, and a decreased number of contigs from 4753 to 267. We subsequently used optical mapping to obtain an accurate whole-genome assembly and placed ~30% of the contigs on eight scaffolds, covering ~94% of the total assembly (34 Mb) (Supplemental Table 3). While the chromosome lengths of the reference strain were found to vary between 3.1 and 6.0 Mb (Klosterman et al. 2011), JR2 chromosome sizes differ and range up to 8.9 Mb (Supplemental Table 4). Thus, despite the observation that the two strains are 99.98% identical in all genomic regions that could be aligned, the karyotype of these two strains is completely different. This observation urged us to investigate collinearity between the chromosomes of the two strains.

Unanticipated, pairwise alignments to identify collinear (synteny) blocks between the two strains revealed repeated interruptions of syntenic regions by intra- and interchromosomal rearrangements (Fig. 2). In total, 11 intra- and 17 interchromosomal rearrangements were identified between the two strains (Fig. 2A,B). While most of these breakpoints were associated with assembly gaps due to the occurrence of repeats in the flanking genomic areas (Fig. 2B,C; Supplemental Table 6), three breakpoints could be pinpointed to the nucleotide exactly, and thus could be experimentally confirmed with PCR (Fig. 1; Supplemental Fig. 1; Supplemental Tables 5, 6). We subsequently extended the analysis of chromosomal rearrangements to all sequenced strains by screening for breakpoints within alignments of the assembled contigs for each of the strains to the reference strain. Although identification of such syntenic breakpoints in the small insert library genome assemblies is hampered by the short contig lengths, three to eight synteny breakpoints could be identified in silico for

Table 1. Summary of SNPs when compared with *V. dahliae* reference strain VdLs17

Strain	#SNPs	#Unique SNPs	#SNPs in intergenic region	#SNPs in introns	#SNPs in exons	d_N[a]	d_S[a]
JR2	5,445	947	3,193	636	1,563	1,092	444
CBS381.66	117,364	166	67,640	8,775	39,062	16,702	21,974
St14.01	117,704	274	67,894	8,786	39,161	16,744	22,030
St.100	163,602	81061	94,516	12,108	54,219	23,350	30,361
DVD-3	118,528	211	68,773	8,716	39,135	16,662	22,098
DVD-31	119,025	316	68,973	8,786	39,381	16,827	22,170
DVD-161	117,277	178	67,831	8,656	38,776	16,526	21,879
DVD-S26	117,307	235	67,653	8,764	39,079	16,743	21,947
DVD-S29	122,057	5552	70,801	9,204	40,219	17,175	22,647
DVD-S94	119,060	205	68,993	8,782	39,303	16,763	22,161
All	236,785	89,145	136,705	17,421	78,342	34,007	43,563

[a]Number of nonsynonymous (d_N) and synonymous substitutions (d_S).

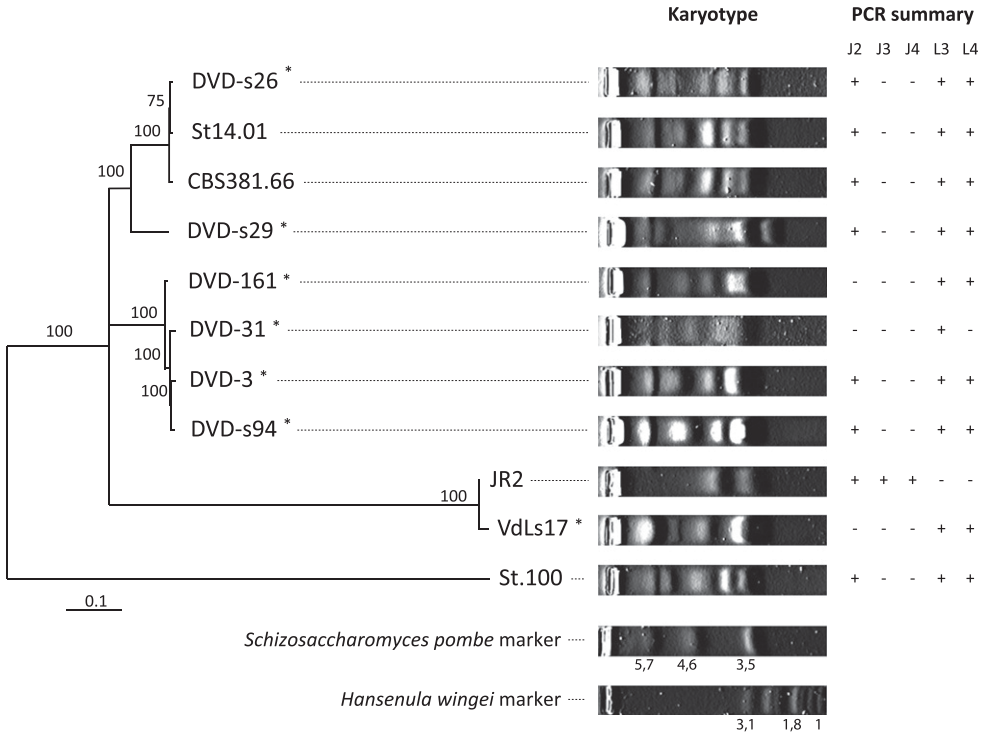

Figure 1. Population structure and chromosome karyotype of sequenced *Verticillium dahliae* isolates. An unrooted maximum likelihood phylogenetic tree based on concatenation of 236,785 SNP sites relative to reference strain VdLs17 is shown. Evolutionary distances based on the Jukes-Cantor method and bootstrap support (%) are indicated at the nodes. Race 2 strains are indicated by asterisks. Pulsed-field gel electrophoresis of sequenced *V. dahliae* strains are shown as well as the results of PCR analyses on structural rearrangements SR2, SR3, and SR4 using JR2-specific primer sets (J2, J3, J4) and VdLs17-specific primer sets (L3, L4), highlighting significant chromosome length and structure polymorphisms between isolates. Chromosomal DNA of *Schizosaccharomyces pombe* and *Hansenula wingei* were loaded as size makers.

each of these strains when compared with the VdLs17 reference genome, which roughly occur in the regions of the previously identified breakpoints between VdLs17 and JR2. The occurrence of several of these breakpoints was confirmed with PCR (Fig. 1; Supplemental Fig. 1). Moreover, pulsed-field gel electrophoresis confirmed considerable chromosome length polymorphism between all strains (Fig. 1) and demonstrates that intra- and interchromosomal rearrangements frequently occur within the genomes of *V. dahliae* strains despite their high degree of sequence conservation.

Particularly, retrotransposons have been implicated in genome rearrangements through homologous recombination between elements or by causing chromosomal breaks during excision or insertion (Mieczkowski et al. 2006; Maxwell et al. 2011). To investigate a potential involvement of retrotransposons in the genomic rearrangements of *V. dahliae*, we identified all repetitive elements in the genome sequences of strains VdLs17 and JR2, and found that both genomes contain only a low-repeat content of ~4% (Klosterman et al. 2011; Supplemental Table 7). Of the different classes of repeats, we only observed significant correlation ($P < 0.01$) between synteny breakpoints and long terminal repeat (LTR) retrotransposons (Supplemental Tables 8–11).

The pan-genome of *V. dahliae*: Lineage-specific regions

In addition to the core ~30 Mb genome, all strains carried up to ~4 Mb of genome sequence that was unique or shared by only a subset of strains, composing a highly dynamic lineage-specific (LS) region of the genome encoding up to 1000 genes (Table 2; Klosterman et al. 2011), which is enriched for various types of transposable elements (Fig. 2B,C; Klosterman et al. 2011; Amyotte et al. 2012). These LS genomic regions are correlated ($P < 0.01$) with syntenic breakpoints in VdLs17 and JR2 (Supplemental Tables 6, 12). In VdLs17 as well as JR2 these regions are typically devoid of housekeeping genes that are required for the maintenance of basic cellular functions such as transcription and translation (Klosterman et al. 2011). Intriguingly, the *Ave1* gene is located within the ~4-Mb LS region of race 1 strains, suggesting that these regions contribute to niche adaptation, i.e., pathogenicity on plant hosts (de Jonge et al. 2012).

To further establish the role of LS regions in *V. dahliae* pathogenicity, we mined these regions, as well as the core genomic regions in VdLs17 and JR2, for candidate effectors (i.e., small, secreted proteins of unknown function). Typically, effector genes encode small proteins that are characterized by the presence of an N-terminal signal peptide that can be predicted computationally (de Jonge 2012). However, we did not observe a remarkable difference in the number of small secreted proteins between the core and LS genomic regions (Table 2). Similarly, no remarkable difference was found in the number of protein domains classified in the Pfam database (Finn et al. 2008). In a number of plant pathogenic oomycetes it was observed that effector genes are found in repeat-rich, gene-sparse regions that can be visualized by plotting the length of the 5′- and 3′-flanking intergenic regions (Raffaele et al. 2010). In the case of *V. dahliae*, we did not find a significant difference in gene density between core and LS genomic regions (Table 2; Sup-

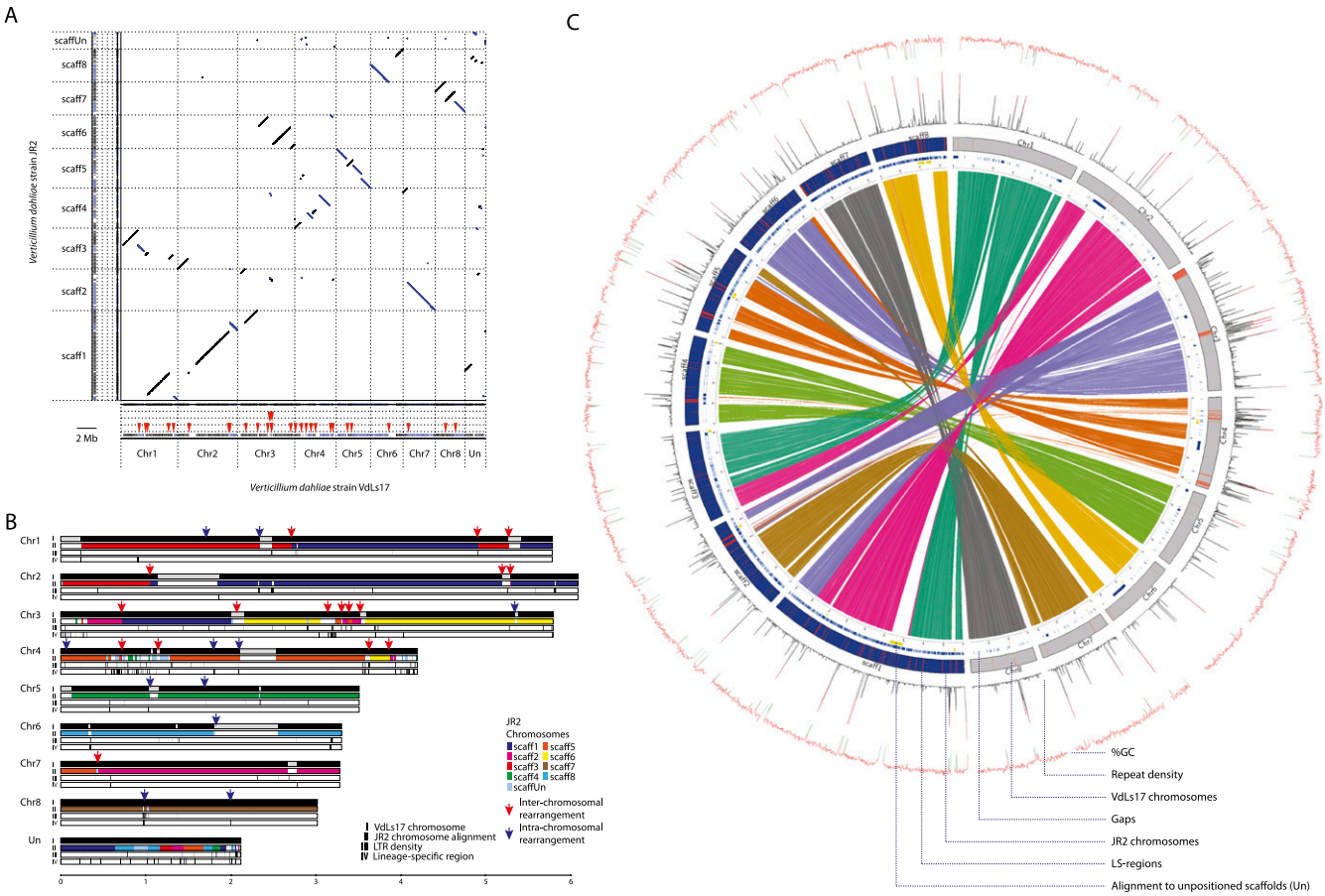

Figure 2. Whole-genome alignment of *Verticillium dahliae* strains VdLs17 and JR2 reveals extensive chromosomal rearrangements. (*A*) Whole-genome dot-plot comparison with forward–forward alignments (black) and inversions (blue). Red triangles mark syntenic breakpoints. (Un) Unplaced contigs during optical mapping. (*B*) Global view of synteny alignments between *V. dahliae* strains VdLs17 and JR2 and the distribution of LTR retrotransponsons and lineage-specific regions. VdLs17 chromosomes are shown as reference. For each chromosome, row I represents genomic scaffolds (black) on the chromosome separated by scaffold breaks (gray); row II displays syntenic alignment of JR2 chromosomes, highlighting 28 major intra- and interchromosomal rearrangements that are marked by red (inter) and blue (intra) arrows *above* each breakpoint; row III represents the density of LTR retrotransposable elements calculated in 10-kb windows; and row IV depicts lineage-specific regions that are found in only a subset of strains. (*C*) Circos diagram illustrating collinear blocks with alignments between VdLs17 (gray) and JR2 (blue) chromosomes, sequence gaps, sequences aligning to unpositioned scaffolds (yellow), lineage-specific sequences (red), repeat density (% coverage of 10-kb window), and GC % (per 10-kb window).

plemental Fig. 2). However, when assessing transcription (RNA-seq) data of *V. dahliae*-infected *N. benthamiana* (de Jonge et al. 2012; Faino et al. 2012), we identified 19 *V. dahliae* genes ($P < 0.001$) that were induced to the same level, or higher, as the previously characterized *Ave1* effector gene [$\log_{10}$(FC) > 2.15; 8-d post-inoculation] when compared with expression in in vitro-cultured mycelium. Remarkably, eight of the 19 genes are located within the ~3-Mb LS region, while the remaining 11 genes reside in the ~30-Mb core genome, identifying a significant overrepresentation of in planta-induced genes in the LS regions (Fig. 3). Of the eight highly

Table 2. Summary of genome size and gene numbers for each sequenced strain, split by the core and lineage-specific (LS) genome

	Genome size (Mb)					Number of genes						(%) Secretome	
Strain	Total	Excl. gaps	Repeats	Core	LS	Total	Secreted	Core	LS	Core/secreted	LS/secreted	Core	LS
St.14.01	34.3	33.9	2.3	30.4	3.4	9,854	979	9,341	513	941	38	10.1	7.4
DVD-3	33.8	33.3	1.9	30.5	2.8	9,881	972	9,335	546	937	35	10.0	6.4
CBS381.66	34.5	34.2	2.6	30.4	3.8	9,834	1,005	9,230	604	961	44	10.4	7.3
St.100	35.0	34.6	0.8	30.1	4.5	9,822	976	9,193	629	916	60	10.0	9.5
JR2	37.5	33.5	1.5	30.1	3.4	10,985	1,085	10,098	887	1,005	80	10.0	9.0
DVD-s94	34.6	34.2	2.6	30.5	3.8	9,846	989	9,289	557	951	38	10.2	6.8
DVD-31	33.8	33.4	2.4	30.4	3.0	9,867	985	9,505	362	951	34	10.0	9.4
DVD-s26	35.0	34.6	2.3	30.4	4.2	10,011	970	9,336	675	930	40	10.0	5.9
DVD-s29	33.7	33.1	2.0	30.2	2.9	9,773	969	9,275	498	939	30	10.1	6.0
DVD-161	33.8	33.4	2.2	30.4	3.0	9,852	997	9,427	425	967	30	10.3	7.1
VdLs17	36.9	33.0	1.3	29.2	3.7	10,535	1,055	9,530	1,005	974	81	10.2	8.1

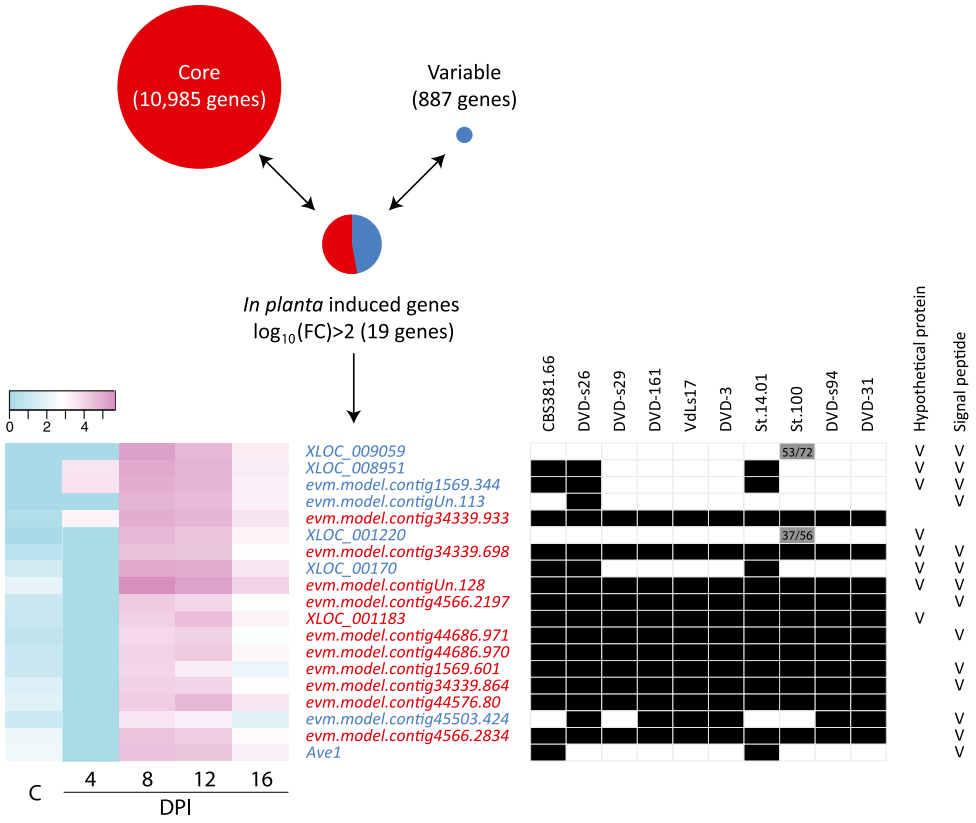

Figure 3. Lineage-specific genomic regions of *Verticillium dahliae* strain JR2 are enriched for in planta-expressed genes. Deep transcriptome sequencing of infected *Nicotiana benthamiana* plants harvested between 4- and 16-d post-inoculation (DPI), and fungus cultured on Czapek Dox medium (C) led to the identification of 19 highly in planta-induced genes [$\log_{10}$(FC) > 2.15]. Of these, eight are located in lineage-specific (LS) regions (in blue), while 11 are in the core genome (in red), demonstrating significant overrepresentation of in planta-expressed genes in LS regions. The table shows the presence (black) or absence (white) of in planta-induced JR2 genes within the set of sequenced strains and indicates hypothetical and putatively secreted proteins. Numbers in the two gray cells indicate amino acid identity and conservation, respectively, for two divergent homologs.

induced genes that reside in LS regions, five (including *Ave1*) encode putative secreted effectors (Fig. 3). Moreover, considering a $\log_{10}$(FC) > 3 or $\log_{10}$(FC) > 4, these ratios are even more skewed with six out of 14 and five out of six in planta-induced genes residing in LS regions, respectively.

Lineage-specific genomic regions determine *V. dahliae* virulence and niche adaptation

To assess the biological significance of the significant overrepresentation of in planta-induced genes in the LS regions, we pursued targeted deletion of two randomly chosen effector genes of the four effector gene candidates in addition to *Ave1* that are most highly expressed in planta; *XLOC_009059* and *XLOC_008951*. *XLOC_009059* does not occur in any of the other strains except for St.100, which contains a highly diversified copy (53% and 72% amino acid identity and conservation, respectively, as determined by BLASTp) (Fig. 3) that is unlikely to encode a functional protein due to the high number of predicted stop codons. On the other hand, *XLOC_008951* was found in four of the 11 strains that were sequenced (Fig. 3). Remarkably, deletion of each of them resulted in significantly reduced disease development, confirming that they encode bona fide effectors required for virulence on tomato (Fig. 4A,B). Thus, we conclude that the LS regions of strain JR2 significantly contribute to virulence.

Similar to JR2, strain VdLs17 also contains a ~4-Mb LS region (Table 2; Klosterman et al. 2011) that was assessed for unique effector genes crucial for pathogenicity. All *V. dahliae* strains that were sequenced contain six genes that encode LysM effectors within their core genome (*VDAG_00902*; *VDAG_03096*; *VDAG_04781*; *VDAG_06426*; *VDAG_06998*; *VDAG_08171*). LysM effectors have previously been implicated in sequestration of chitin fragments to prevent these fragments from stimulating host immunity (de Jonge and Thomma 2009; de Jonge et al. 2010; Marshall et al. 2011; Mentlak et al. 2012). VdLs17 contains an additional LysM effector gene (*VDAG_05180*) within a LS region, encoding a LysM effector that contains two LysM domains. Remarkably, of the seven LysM effectors in the VdLs17 genome, only this LysM effector gene is significantly expressed in planta (Fig. 4C). Furthermore, targeted deletion of this effector revealed that it is indeed required for virulence of the VdLs17 strain (Fig. 4D,E). Thus, like in JR2, the LS regions of strain VdLs17 contribute to virulence. Notably, like the race-1-specific effector *Ave1* (de Jonge et al. 2012), *VDAG_05180* as well as *V. dahliae* JR2 effector genes *XLOC_009059*, *XLOC_008951* and the candidate effector genes *XLOC_00170* and *evm.model.contigUn.128* are flanked by repetitive elements such as LTR retrotransposons and FotI transposases (Fig. 5; Supplemental Figure 3), suggesting that the presence/absence polymorphism of all of these effector genes in *V. dahliae* is mediated by the flexibility and instability of the genomic context as a consequence of

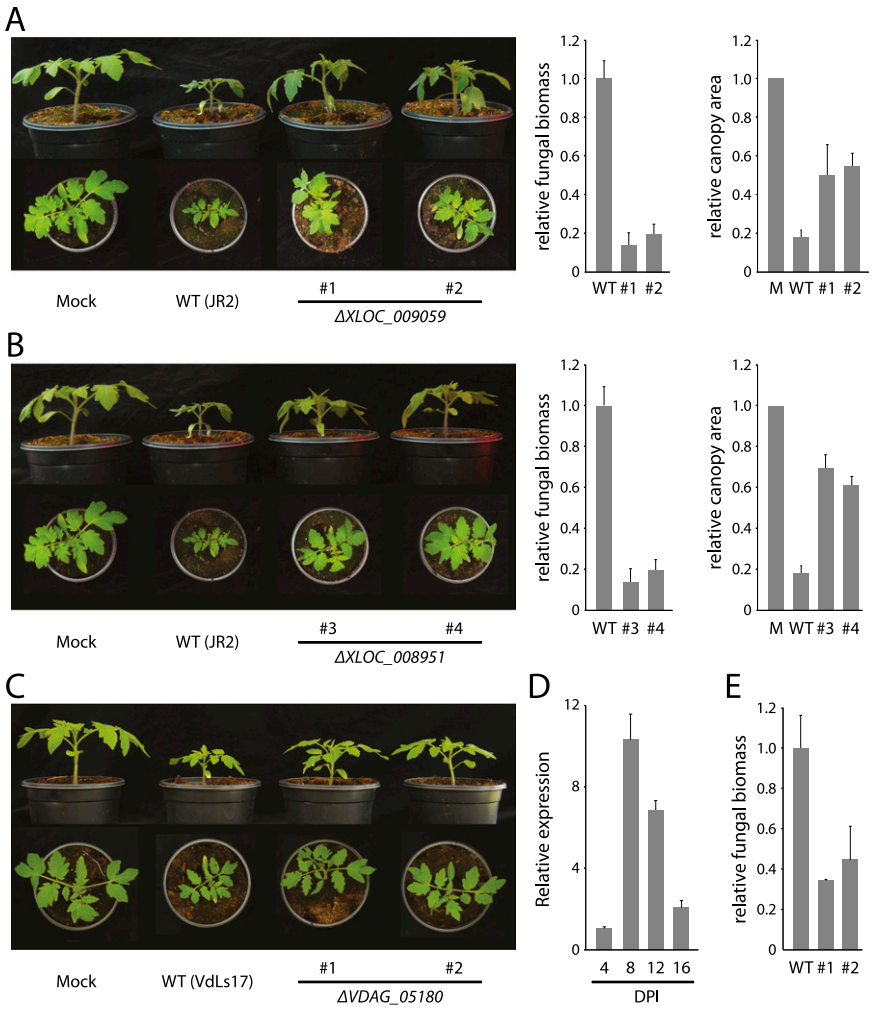

Figure 4. Lineage-specific genomic regions of *Verticillium dahliae* harbor effectors that are required for virulence on tomato. (*A*) Two independent *XLOC_009059* deletion strains (*ΔXLOC_009059*) show compromised virulence on tomato (cv. MoneyMaker), evidenced by reduced stunting when compared with inoculation with wild-type *V. dahliae* (WT, JR2), increased canopy area, and reduced *V. dahliae* biomass accumulation. (*B*) Two independent *XLOC_008951* deletion strains (*ΔXLOC_008951*) show compromised virulence on tomato (cv. MoneyMaker), evidenced by reduced stunting when compared with inoculation with wild-type *V. dahliae* (WT, JR2), increased canopy area, and reduced *V. dahliae* biomass accumulation. (*C*) Two independent *VDAG_05180* deletion strains (*ΔVDAG_05180*) show compromised virulence on tomato (cv. Motelle), evidenced by reduced stunting when compared with inoculation with wild-type *V. dahliae* (WT, VdLs17). Photographs are taken at 12 DPI. (*D*) Expression of *VDAG_05180* in *V. dahliae* strain VdLs17 during infection of *Nicotiana benthamiana* between 4- and 16-d post-inoculation (DPI). (*E*) Reduced *V. dahliae* biomass in plants inoculated with two independent *VDAG_05180* deletion strains when compared with wild-type *V. dahliae* (WT, VdLs17) at 8 DPI. Error bars represent standard error of three replicate experiments.

these repetitive elements (Klosterman et al. 2011; Amyotte et al. 2012).

Discussion

So far, it remains unknown how asexually propagating organisms, including many fungal species, are able to adapt to changing environmental conditions. The lack of sexual reproduction, and consequently meiotic recombination, is thought to greatly affect the potential to generate novel genetic combinations. Especially, pathogenic microbes involved in an intimate interaction with a host need to continuously adapt their effector gene repertoire to counteract host defense responses that have evolved in the evolutionary arms race with the pathogen. Here, we examined the genetic diversity in a population of the plant pathogenic fungus *V. dahliae* that propagates solely through asexual reproduction. As expected for an asexually reproducing organism, we only found a low degree of genome-wide nucleotide diversity between *V. dahliae* strains in the population, ranging between 0.02% and 0.5%. For comparison, in a study on the sexually reproducing fungal wheat pathogen *Zymoseptoria tritici* (synonym of *Mycosphaerella graminicola*), the average genome-wide nucleotide diversity was determined to range between 2% and 4% (Stukenbrock et al. 2011). Despite the low degree of genetic diversity, we identified 29 genes that are under positive selection within the *V. dahliae* population (Supplemental Table 2), suggesting that these genes are under adaptive evolution. This number is comparable to the number of genes under positive selection in *Z. tritici* when compared with its wild sister species (Stukenbrock et al. 2011). However, only three of these *V. dahliae* genes encode secreted proteins that are candidate effectors, suggesting that molecular evolution at the nucleotide level does not play a major role in host adaptation of this asexual pathogen.

Various mechanisms have been described that facilitate rapid development of novel effector genes in pathogenic microbes, including diversity at genomic locations enriched for transposons, mutation, and recombination in subtelomeric regions (McDonagh et al. 2008; Chuma et al. 2011), coregulated gene clusters (Pallmer and Keller 2010; Schirawski et al. 2010), small dispensable chromosomes (Coleman et al. 2009; Ma et al. 2010; Stukenbrock et al. 2010; Goodwin et al. 2011; Raffaele and Kamoun 2012), gene sparse regions (Raffaele et al. 2010), AT-rich isochore-like regions (van der Wouw et al. 2010; Rouxel et al. 2011), genome hybridization (Stukenbrock et al. 2012), and horizontal gene transfer (Friesen et al. 2006; de Jonge et al. 2012; Gardiner et al. 2012). However, most of these mechanisms have been described in species that can reproduce sexually, and of which the genomes were shaped by repeat-driven expansion (Raffaele and Kamoun 2012).

Here we describe the observation of a novel mode of evolution of pathogenicity in the asexual plant pathogenic fungus *V. dahliae*, of which only 4% of the genome is repetitive (Supplemental Table 7; Klosterman et al. 2011; Amyotte et al. 2012). This is significantly less when compared with the genomes of other filamentous pathogens, such as the fungi *Magnaporthe oryzae* (10%), *Z. tritici* (17%), *Fusarium oxysporum* (28%), *Cladosporium fulvum*

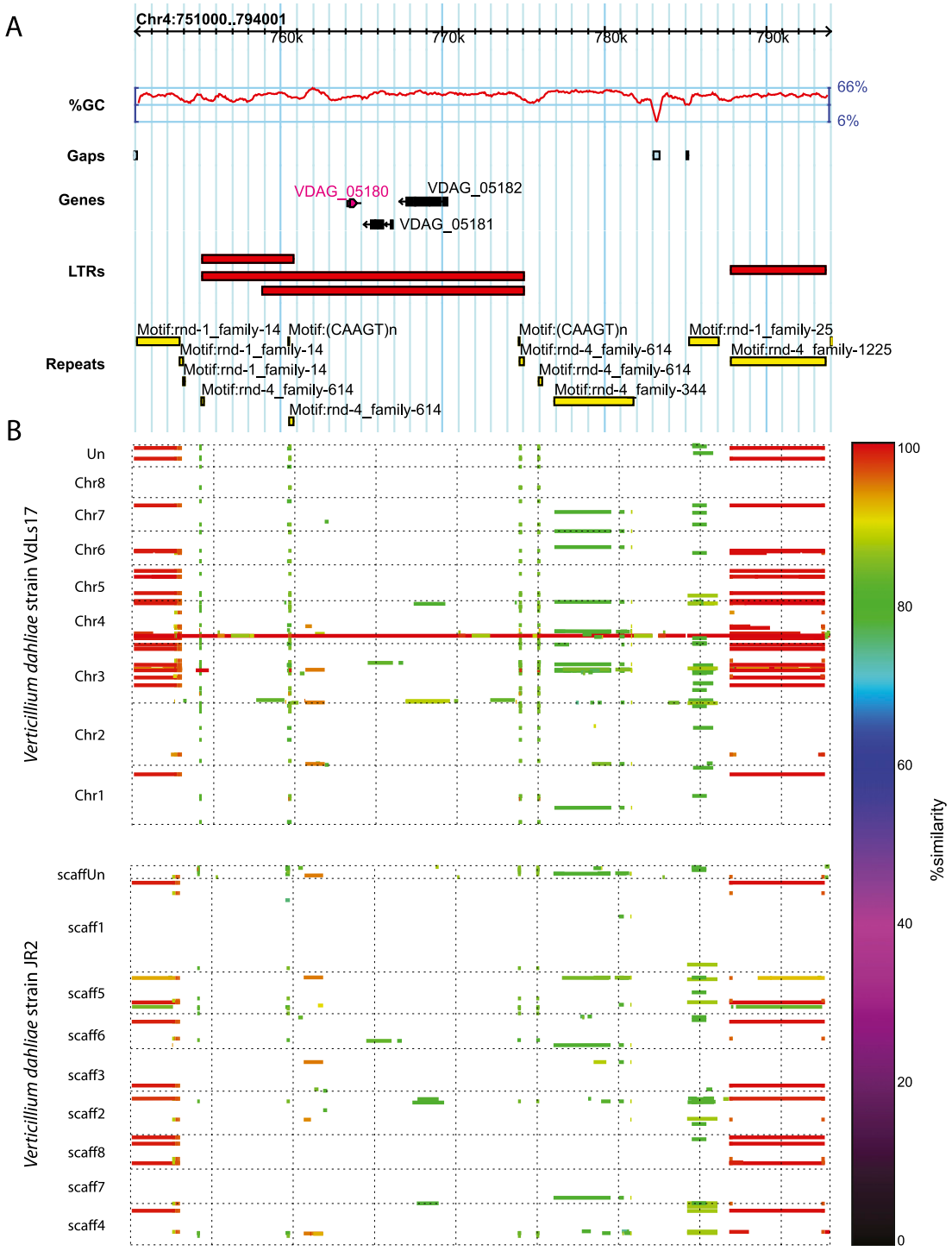

Figure 5. Genomic context of the lineage-specific *Verticillium dahliae* LysM effector *VDAG_05180*. (*A*) Genomic location of *VDAG_05180* in *V. dahliae* strain VdLs17 revealing the presence of flanking gaps, repeats, and a predicted overlapping, but inactive LTR retrotransposon. RepeatModeler families rnd-1_family14, rnd-4_family-614, rnd-1_family-25, and rnd-4_family-1225 are classified as Ty1-Copia, Ty1-Gypsy, TcMar-Fot1, and Ty1-Copia, respectively, by RepeatMasker. (*B*) Repetitiveness of the genomic context of *VDAG_05180* throughout the genomes of VdLs17 (*top*) and JR2 (*bottom*). The degree of conservation is indicated by a color scale, showing recent (red) and more ancient (orange to green) multiplications, especially of repeat elements. In addition, the presence of a *VDAG_05181* homolog (encoding a tetrahydroxynaphthalene reductase) in the JR2 genome, a duplication of this gene in the VdLs17 genome, and the presence of multiple *VDAG_05182* homologs (encoding a Kelch domain-containing protein) in both genomes is shown. The LysM effector gene *VDAG_05180* is uniquely found in the VdLs17 genome.

(47%), *Blumeria graminis* (64%), and the oomycetes *Hyaloperonospora arabidopsidis* (42%) and *Phytophthora infestans* (74%) (Dean et al. 2005; Haas et al. 2009; Baxter et al. 2010; Ma et al. 2010; Spanu et al. 2010; Goodwin et al. 2011; de Wit et al. 2012). Comparative analyses between highly similar *V. dahliae* strains revealed numerous intra- and interchromosomal rearrangements and extensive karyotype variation. This is remarkable, as it is generally assumed that genome rearrangement increases with increasing sequence divergence. Previous analyses of fungal genomes have revealed that although large syntenic regions with orthologous genes arranged in the same orientation across species (known as macrosynteny) are rare, chromosomal gene content is typically preserved among Ascomycetes, particularly in the Dothidiomycete class (Goodwin et al. 2011; Hane et al. 2011). Such preservation of chromosomal gene content without conservation of gene order or orientation is referred to as mesosynteny, and is characterized by the occurrence of numerous intrachromosomal rearrangements, presumably arising as the consequence of frequent inversions during meiosis rather than interchromosomal rearrangements (Hane et al. 2011). The extensive number of interchromosomal rearrangements between *V. dahliae* isolates indicates that mesosynteny is not crucial in *V. dahliae*.

Unlike previous observations in a number of fungal plant pathogens, including *F. oxysporum*, *N. haematococca*, and *Z. tritici*, which display karyotype variation as a consequence of the differential presence of small dispensable chromosomes (Coleman et al. 2009; Ma et al. 2010; Stukenbrock et al. 2010; Goodwin et al. 2011; Raffaele and Kamoun 2012), the karyotype variation that we observed between individual isolates of *V. dahliae* concerns chromosome length polymorphisms, and no indication for the occurrence of small dispensable chromosomes is found in *V. dahliae*. The observed variation in this study and previous ones among fungal karyotypes (Zolan 1995) is the result of complex chromosomal rearrangements. It has previously been suggested that karyotype variation, rather than being a mechanism of adaptation to generate novel virulence traits, occurs because nondeleterious genomic rearrangements are maintained due to the absence or rarity of a sexual cycle (Talbot et al. 1993; Zolan 1995). Our data challenge this hypothesis by showing that chromosomal plasticity, evidenced by extensive targeted chromosomal rearrangements and karyotype variability, is genetically non-neutral as it induces local sequence variation at syntenic breakpoints and increases adaptive capability, as demonstrated by the significant enrichment for novel effector genes and acquisition of the race 1-specific effector *Ave1*, possibly through horizontal gene transfer and subsequent loss in *V. dahliae* race 2 isolates (de Jonge et al. 2012). Moreover, our data reinforces the notion that *V. dahliae* strictly relies on asexual reproduction, as extensive karyotype variation prevents correct pairing of homologous chromosomes during meiosis (Kistler and Miao 1992).

Our phylogenetic analysis based on whole-genome sequence data (Fig. 1) has demonstrated that race 1 and race 2 isolates of *V. dahliae* do not form separate clades, and the fact that we did not observe any sequence variation for *Ave1* within and between *Verticillium* species (de Jonge et al. 2012) strongly suggests that *Ave1* has been acquired (in race 1) or lost (in race 2) several times. Similarly, we identified additional effector genes within the plastic genomic regions that are unique or shared by only a subset of strains and like *Ave1*; these are conserved and not monophyletic, suggesting that they are frequently lost and/or readily exchanged between individual strains as has been proposed for effector genes of the *Avr-Pita* family in *M. oryzae* (Chuma et al. 2011). We speculate that frequent presence/absence polymorphisms of effector genes in these plastic regions is mediated by the flexibility and instability of these regions, reflected by the enrichment for transposable elements and association with chromosomal rearrangements (Amyotte et al. 2012). Moreover, we note that particular transposable elements flank a number of the in planta highly induced effector genes located in the LS regions and speculate that, besides providing genetic flexibility, these elements might also affect the level of gene expression. An association between in planta-expressed effector genes and a particular transposable element was recently found for the tomato pathogenic vascular wilt fungus *Fusarium oxysporum* f. sp. *lycopersici*, in which a repetitive *miniature Impala* (mimp) element was found to be present in the promoter region of all *SIX* (secreted in xylem) effector genes (Schmidt et al. 2013). Similarly, members of the *M. oryzae Avr-Pita* effector gene family are flanked by a retrotransposon, presumably contributing to its translocation across the genome (Chuma et al. 2011). Presence/absence of polymorphism and translocation of effector genes that are associated with unstable genomic regions has also been observed in asexual lineages of *M. oryzae* (Yoshida et al. 2009; Chuma et al. 2011), and it was hypothesized that parasexual recombination facilitates the exchange of effector genes between these lineages (Noguchi et al. 2006; Chuma et al. 2011). Parasexual recombination involves fusion of haploid fungal hyphae, followed by the formation of diploid nuclei through hybridization that can later return to a haploid state. Mitotic recombination may result in nonsexual exchange of genetic material without meiosis. In particular cases, the hybridized nuclei are stable, resulting in a hybrid species with a doubled genome such as has been suggested for *V. longisporum* (Inderbitzin et al. 2011). Parasexuality has been identified in many, mostly asexually propagating fungi during in vitro culturing and was shown to contribute to adaptive capabilities of the model fungus *Aspergillus nidulans* in vitro (Schoustra et al. 2007). Parasexual recombination may also be involved in the exchange of effector genes between asexual lineages of *V. dahliae*. Laboratory studies have shown that transgenic *V. dahliae* strains that carry auxotrophic markers are capable of hyphal fusion and exchange of genetic material, depending on their vegetative compatibility (Hastie 1964, 1973). Likewise, transfer of pathogenicity chromosomes between asexual lineages of *F. oxysporum* may be mediated by parasexual cycles (Corell 1991; Ma et al. 2010). However, the significance of parasexuality in nature, and the genetic factors that control parasexual compatibility between lineages, are poorly understood, although evidence for parasexual recombination in field populations of *M. oryzae* has been reported (Zeigler et al. 1997; Glass et al. 2000).

In our study, chromosomal rearrangements were found to be frequently associated with retrotransposons, a class of repetitive sequence elements that is known to promote genome instability through recurrent excision and insertion as well as by mediating homologous recombination during cell division (Zolan 1995; Lemoine et al. 2005; Maxwell et al. 2011). In organisms without a cycle of sexual reproduction, such as *V. dahliae*, homologous recombination between highly similar copies of transposable elements might help to generate genetic diversity by facilitating mitotic crossover, thereby creating new genetic combinations that are potentially beneficial. In addition, altered cosuppression, silencing of dispersed homologous genomic regions through directed DNA methylation, might act on proliferation of transposable elements to affect genome stability (Martienssen and Colot 2001; Selker et al. 2003; Muszewska et al. 2011). Although it is generally believed that asexual reproduction limits genetic variation, and consequently

limits adaptive capability of the organism, we provide evidence for chromosomal plasticity as facilitator of asexual genome evolution.

Methods

SNP identification

Illumina reads (de Jonge et al. 2012) were mapped onto the *Verticillium dahliae* VdLs17 reference genome using GSNAP (version 2012-04-16; Wu and Nacu 2010) with default settings and processed by Picard (version 1.57; http://picard.sourceforge.net). SNPs were identified by SAMtools mpileup (version; 0.1.18; http://samtools.sourceforge.net) and filtered using vcfutils.pl varFilter (-Q20, -D100) and a minimum allele frequency of 0.8. SNP statistics were determined by VCFtools (version 0.1.10; Danecek et al. 2011) and variant annotation by SnpEff (version 2.1; http://snpeff.sourceforge.net). To specifically identify genes that are subject to positive selection, we applied the Nei Gojobori method, where rates of K_a and K_s are compared using a *Z*-test (Nei and Kumar 2000; Stukenbrock and Dutheil 2012). *Z*-values >2326 are considered significant with $P < 0.01$.

Coverage breadth was calculated for all genes as the percentage of nucleotides with minimum one read aligned using BEDtools (version 2.16.1; Quinlan and Hall 2010). Genes were considered absent when breadth was <0.1.

Maximum likelihood phylogenetic analysis was performed in MEGA5 (Tamura et al. 2011). For each strain, as input we extracted for all nonredundant SNP positions the respective base call, resulting in 11 sequences, each containing 236,785 bp.

Assembly, alignment, and identification of rearrangements

Draft assemblies generated previously (de Jonge et al. 2012) were used for all strains, except for JR2. Mate-pair library preparation and sequencing (50 bp, ~5-kb insert) of JR2 was performed by the BGI (Hong Kong). For de novo assembly, Velvet (version 1.2.03; Zerbino and Birney 2008) was used with cov_cutoff=6, exp_cov=auto, k-mer=31 and shortMatePaired=yes to generate a new assembly based on the 500-bp paired-end and 5-kb mate-pair libraries. Optical mapping, i.e., the construction of ordered genome-wide, high-resolution (>150×) restriction maps, was performed by BGI using the Argus System, and contigs were placed using MapSolver (version 3.2; OpGen).

Whole-genome alignments and dot plots were generated by MUMmer 3.0 (Kurtz et al. 2004) using NUCmer with default settings (except for -l 15 and–maxmatch) and Mummerplot. We used custom scripts to identify rearrangements and associated breakpoints. Core and LS genomic regions were determined by assessing coverage breadth of the alignments on 1-kb nonoverlapping windows using BEDTools. In addition, we determined the percentage of gaps for each region. Regions were considered lineage specific with an alignment breadth of <0.2. Core genomic regions were defined based on their minimum presence in all strains except one in order to compensate for assembly and alignment inconsistencies. Similarly, genes were considered as core genes when identified minimally in all strains except one with an alignment breadth of >0.8 for each strain. Remaining genes are considered lineage specific. Collinear blocks, repetitiveness, percentage GC, and LS genomic regions in the VdLs17 and JR2 genomes were visualized using Circos (version 0.55; Krzywinski et al. 2009) and GBrowse (version 2.49; Stein et al. 2002).

Multiple chromosomal rearrangements were verified by PCR. To this end, primer pairs spanning predicted breakpoints were designed for JR2 and VdLs17 to selectively amplify breakpoint regions in either of the two genotypes and used in PCR reactions on genomic DNA. Quality of input DNA and integrity of syntenic sequences flanking the breakpoints in JR2 and VdLs17 were verified by control primer sets (Supplemental Table 5).

Repeat identification

Repetitive elements were identified and classified by RepeatModeler (version 1.0.5; http://www.repeatmasker.org/RepeatModeler.html), incorporating the two de novo repeat-finding programs RECON (version 1.0.7; Bao and Eddy 2002) and RepeatScout (version 1.0.3; Price et al. 2005), the tandem repeat finder TRF (version 3.2.1), and the annotated repeat library from RepBase (release 16.12). Repetitive sequence families were used by RepeatMasker (version open-3.3.0; http://www.repeatmasker.org) to identify and mask all repetitive sequences applying the sensitive mode. Full-length long terminal repeat (LTR) retrotransposons were identified by LTR_FINDER (version 1.0.5; Xu and Wang 2007). Statistical significant spatial correlations between repetitive sequences, breakpoints, and genomic regions were assessed by GenometriCorr (version 1.1.7; http://genometricorr.sourceforge.net).

Karyotyping

V. dahliae mycelium was prepared for protoplasting following the mycelium-based fungal biomass preparation method (Mehrabi et al. 2012). Mycelium was digested in 1 M sorbitol containing 1% (w/v) glucanex and 0.5% driselase (Sigma-Aldrich) at 32°C until protoplast concentration reached 10^8/mL. Protoplast plugs were prepared and stored as described (Mehrabi et al. 2012). Karyotyping was carried out using a CHEF Mapper XA pulsed field electrophoresis system (Bio-Rad) using the auto algorithm function with low- and high-molecular weight settings at 2 and 6 Mb, respectively. Chromosomes were separated in 0.8% low EEO agarose (US Biological) gels. Chromosome size markers from *Hansenula wingei* and *Schizosaccharomyces pombe* (Bio-Rad) were included as reference. After electrophoresis, gels were stained with ethidium bromide (1 μg/mL) in water for 1 h and destained in water for 2 h.

Gene prediction, expression, and annotation

Gene predictions on the genome sequences of all *V. dahliae* strains were performed using a combinatorial approach, applying the EVidenceModeler (version r2012-06-25; Haas et al. 2008) to combine protein-coding gene evidence from *V. dahliae*-trained Augustus (version 2.6; Stanke et al. 2006, 2008), GeneMarkES (version 2.3c; Borodovsky and Lomsadze 2011), *V. dahliae*-trained GlimmerHMM (version 3.0.1; Majoros et al. 2004), protein alignments to the latest *Fusarium graminearum* (release 3.2, MIPS, Munich) (Wong et al. 2011) and *Magnaporthe oryzae* (release 8, Broad Institute) proteins using the Analysis and Annotation Tool (AAT) package (version r03052011; Huang et al. 1997). For deep transcriptome sequencing and mapping, ~2 Gb of reads from *V. dahliae* strain JR2-infected *Nicotiana benthamiana* plants and ~1 Gb of reads from *V. dahliae* JR2 cultured in vitro on Czapek Dox medium were mapped on the *V. dahliae* JR2 genome using TopHat (version 2.0.8; Trapnell et al. 2009; de Jonge et al. 2012). Cufflinks (version 2.0.2; Trapnell and Salzberg 2010) was used to assemble novel transcripts and isoforms from the mapped reads that were subsequently incorporated in the final gene-set for JR2 using the EVidenceModeler after manual curation. Relative expression for each gene in each experiment was determined by assessing the number of reads mapping to each gene using HTSeq (version 0.5.4p1; www-huber.embl.de/users/anders/HTSeq/) applying the default "union" setting and subsequently reported in the number of Reads Per Kilobase of transcript per Million mapped reads (RPKM). For differential gene expression

analysis we compared in planta and in vitro expression using Excel and CummeRbund (http://compbio.mit.edu/cummeRbund/). The predicted proteins were mined for candidate effectors (de Jonge 2012) by functional annotation using BLASTp analyses against the nonredundant database at NCBI (http://www.ncbi.nih.nlm.gov) and Pfam domain scanning (Finn et al. 2008) using the Blast2GO suite (Conesa et al. 2005). Secreted proteins were predicted by SignalP4 (Petersen et al. 2011). Gene density was determined as previously described (Raffaele et al. 2010). In short, 5′- and 3′-flanking intergenic regions were calculated, scored in two-dimensional bins, and plotted. Filled contour plots were generated using R (http://www.r-project.org/).

Functional analysis

Knockouts were generated by amplifying the sequences flanking coding sequences using the primer sets KO-XLOC_009059-F1/KO-XLOC_009059-R1 and KO-XLOC_009059-F2/KO-XLOC_009059-R2 for *XLOC_009059*, KO-XLOC_008951-F1/KO-XLOC_008951-R1 and KO-XLOC_008951-F2/KO-XLOC_008951-R2 for *XLOC_008951*, and KO-VDAG_05180-F1/KO-VDAG_05180-R1 and KO-VDAG_05180-F2/KO-VDAG_05180-R2 for *VDAG_05180* (Supplemental Table 4). Likewise, knockouts of *XLOC_009059* and *XLOC_008951* were generated by amplification of the flanking sequences (Supplemental Table 4). PCR products were subsequently cloned into pRF-HU2 (Frandsen et al. 2008). *V. dahliae* transformation and subsequent inoculations on tomato (cv. Motelle and MoneyMaker) plants to assess the impact on virulence were performed as described (Fradin et al. 2009). Plants were regularly inspected during a 2-wk interval and photographed at 8- and 12-d post -inoculation (DPI). For biomass quantification, the roots and stem below cotyledons of three plants per *V. dahliae* genotype were flash-frozen in liquid nitrogen. To determine canopy surface areas, we measured the leaf surface areas of multiple plants from photographs taken from the top using ImageJ. The samples were ground to powder, of which an aliquot was used for DNA isolation (Fulton et al. 1995). Real-time PCR was conducted with primer sets SlRub-F1/SlRub-F2 for tomato *RuBisCo* and VdGAPDH-F/VdGAPDH-R for *V. dahliae GAPDH* (Supplemental Table 4). For expression analyses, 3-wk-old *Nicotiana benthamiana* plants were inoculated with strain VdLs17 as previously described (Fradin et al. 2009), harvested at 4, 8, 12, and 16 DPI, and flash-frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Kit (Qiagen) and cDNA was synthesized by SuperScript III (Invitrogen). Real-time PCR was conducted with primer sets VdGAPDH-F/VdGAPDH-R for *V. dahliae GAPDH* and qVDAG_05180-F1/qVDAG_05180-R1 for *V. dahliae VDAG_05180* (Supplemental Table 4).

Data access

All genome and transcriptome raw read data have been deposited at the NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under BioProject PRJNA169154 (http://www.ncbi.nlm.nih.gov/bioproject). The *V. dahliae* strain JR2 draft genome sequence is deposited at the NCBI WGA archive database (http://www.ncbi.nlm.nih.gov/genome) under accession number APFD00000000 and described under BioProject PRJNA175765.

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