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A 20-kb lineage-specific genomic region tames virulence in pathogenic amphidiploid *Verticillium longisporum*

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Abstract

Amphidiploid fungal *Verticillium longisporum* strains Vl43 and Vl32 colonize the plant host *Brassica napus* but differ in their ability to cause disease symptoms. These strains represent two *V. longisporum* lineages derived from different hybridization events of haploid parental *Verticillium* strains. Vl32 and Vl43 carry same-sex mating-type genes derived from both parental lineages. Vl32 and Vl43 similarly colonize and penetrate plant roots, but asymptomatic Vl32 proliferation in planta is lower than virulent Vl43. The highly conserved Vl43 and Vl32 genomes include less than 1% unique genes, and the karyotypes of 15 or 16 chromosomes display changed genetic synteny due to substantial genomic reshuffling. A 20 kb Vl43 lineage-specific (LS) region apparently originating from the *Verticillium dahliae*-related ancestor is specific for symptomatic

Vl43 and encodes seven genes, including two putative transcription factors. Either partial or complete deletion of this LS region in Vl43 did not reduce virulence but led to induction of even more severe disease symptoms in rapeseed. This suggests that the LS insertion in the genome of symptomatic *V. longisporum* Vl43 mediates virulence-reducing functions, limits damage on the host plant, and therefore tames Vl43 from being even more virulent.

KEYWORDS

genome comparison, hybridization, lineage-specific region, pathogenicity, *Verticillium longisporum*

1 | INTRODUCTION

Plant–fungus interactions are dynamic and require ongoing adaptations of both partners to changing conditions in a coevolutionary process. Fungal success requires a balanced equilibrium with the host's immune system, which limits propagation of the invading fungus and restricts plant damage. Analyses of fungal genomes revealed diverse adaptation mechanisms to new ecological niches and different hosts by changes or additions of genetic information (Seidl & Thomma, 2014).

One mechanism representing evolution-driven genome extension in fungi is genome hybridization. Yeast hybrids have often lost complete or large parts of chromosomes, whereas other genomic regions became homozygous (Morales & Dujon, 2012). Ribosomal and mitochondrial DNA become homogenized and mostly derive from one of the two hybridized species (Morales & Dujon, 2012). Retention of duplicated genes presumably depends on the complexity of the genetic interactions between paralogs. A low degree of entanglement favours diversification of the two genes, which then fulfil different functions and persist (Kuzmin et al., 2020). The evolution of filamentous fungal allodiploids by hybridization contributes to the phenotype diversity of human pathogens such as *Aspergillus lactus* (Steenwyk et al., 2020) and plant pathogens such as *Zymoseptoria pseudotritici* (Stukenbrock et al., 2012) or *Verticillium longisporum* (Inderbitzin, Davis, et al., 2011).

Ascomycetous fungi of the genus *Verticillium* are worldwide distributed vascular plant pathogens. Allodiploid *V. longisporum* is the only known interspecific hybrid of its genus, with *V. dahliae* and *V. alfalfae* (formerly *V. albo-atrum*) as its closest relatives (Clewes et al., 2008; Inderbitzin et al., 2013). At least three separate hybridization events between two distinct haploid parental lineages resulted in this species (Inderbitzin, Davis, et al., 2011; Figure 1a). All known *V. longisporum* lineages evolved from the ancestor A1 (not yet isolated), which hybridized with one of three different partners designated D1 to D3. The D2 and D3 strains are classified as *V. dahliae*, whereas D1 represents another not yet identified ancestor. *V. longisporum* lineages A1/D1 and A1/D3 were isolated in Europe, Japan, and the USA, whereas A1/D2 was exclusively found in the USA (Depotter, Rodriguez-Moreno, et al., 2017; Tran et al., 2013; Zeise & von Tiedemann, 2002). The three hybrids display differences in pathogenicity and are mainly virulent on Brassicaceae (Eynck et al., 2007; Novakazi et al., 2015; Zeise & von Tiedemann, 2002). Lineage A1/D1 is the most pathogenic, whereas lineage A1/D3 is the least able to cause disease on rapeseed.

In contrast to the narrow host range of *V. longisporum*, the relative haploid *V. dahliae* is the main causative agent of Verticillium wilt in about 400 different hosts and generates severe yield losses in important crops (EFSA Panel on Plant Health, 2014). Single *V. dahliae* isolates show altered aggressiveness on different hosts and can colonize plants asymptomatically (Gibriel et al., 2019; Pegg & Brady, 2002; Resende et al., 1994; Zeise & von Tiedemann, 2002). *V. dahliae* isolates rarely infect Brassicaceae and, when found, remain in lower parts of symptomless plants (Eynck et al., 2007; Zhou et al., 2006). Currently, lucerne is the only known host of *V. alfalfae* (Inderbitzin, Bostock, et al., 2011). Hybridizations between genetically distinct parental species gave rise to *Verticillium* spp. with altered host range and virulence (Inderbitzin, Davis, et al., 2011).

Interspecific hybrids can emerge from sexual mating or by vegetative hyphal fusions. Genes required for sexual mating are present in the genomes of *Verticillium* spp., but sexual reproduction has not yet been observed (Milgroom et al., 2014; Short et al., 2014). For sexual compatibility, opposite idiomorphs of the *MAT* locus, the major regulators of sexual recombination in ascomycetes, are required (Debuchy & Turgeon, 2006; Metzenberg & Glass, 1990; Turgeon & Yoder, 2000). Most *V. longisporum* genomes harbour copies of the *MAT1-1* idiomorph, with *MAT1-1-1* and *MAT1-1-3* encoding an α-box transcription factor and an HMG domain protein (Depotter, Seidl, et al., 2017; Inderbitzin, Bostock, et al., 2011). In contrast, 99% of analysed *V. dahliae* isolates harbour the *MAT1-2* gene (Short et al., 2014). The unequal distribution of the *MAT* idiomorphs and the absence of a sexual cycle suggest that sexual reproduction between the two parental species of *V. longisporum* is unlikely. Extensive sequence changes in the *MAT* loci and separation of genetic clusters in population structure analysis also suggest that sexual reproduction in *V. longisporum* is unlikely (Depotter, Seidl, et al., 2017). Another hybridization mechanism is reproduction by hyphal anastomosis followed by nuclear fusion, resulting in duplication of the genome
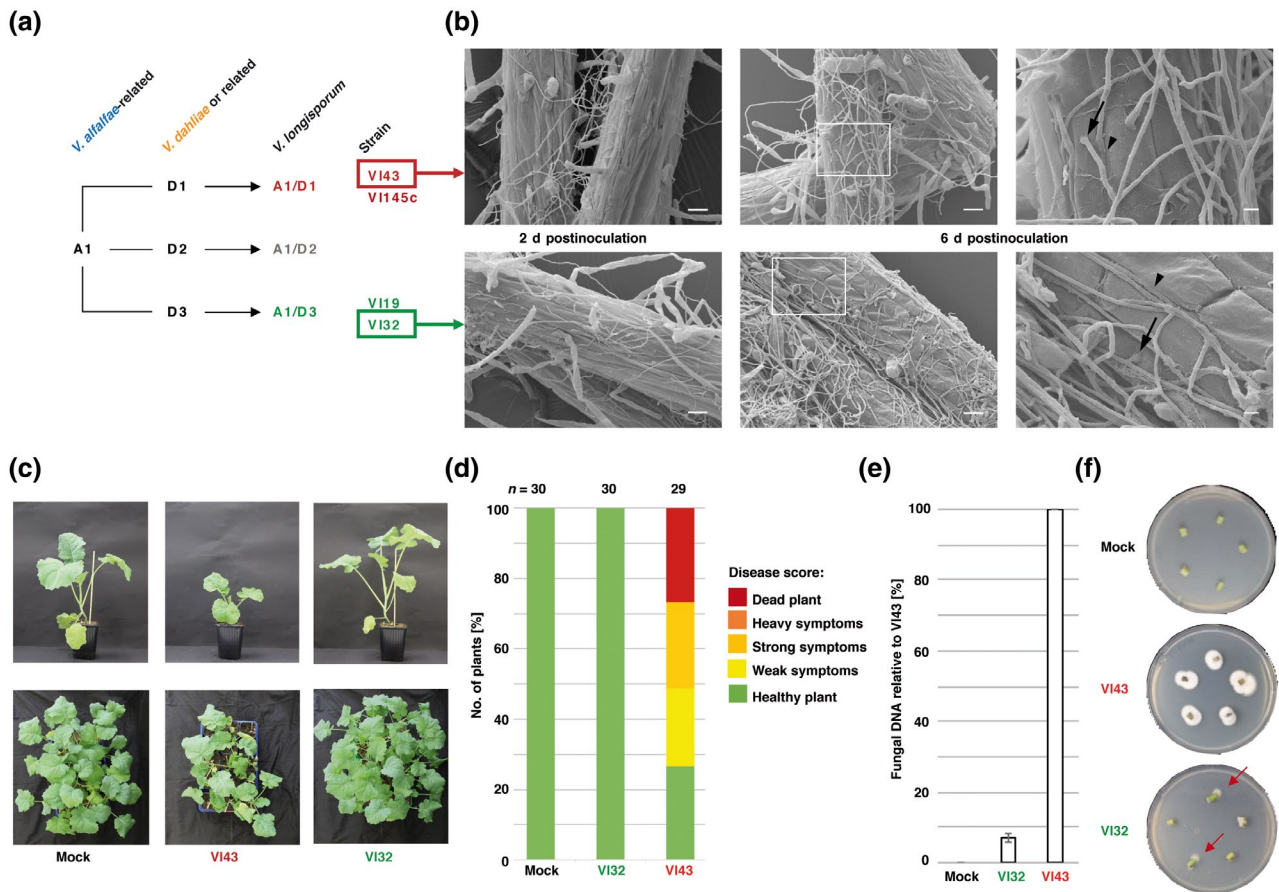
FIGURE 1 *Verticillium longisporum* Vl43 and Vl32 colonize *Arabidopsis thaliana* roots, but only Vl43 induces disease symptoms in rapeseed. (a) Three allodiploid *V. longisporum* lineages emerged from hybridization events of haploid strains. Vl43/Vl145c and Vl32/Vl19 strains originate from hybridization between strains A1 and D1 or D3, respectively (Inderbitzin, Davis, et al., 2011; Tran et al., 2013). (b) Scanning electron micrographs of *V. longisporum* Vl32 or Vl43 colonizing *A. thaliana* roots 2 or 6 days after root dipping into spore solutions. Scale bars: left and middle, 20 μm; right, detailed view of the framed area, 4 μm; arrows: hyphopodia as possible entry sites; arrow heads: hyphae growing at interface of plant cells. (c) and (d) *V. longisporum* strain Vl43, but not Vl32, induces disease symptoms in *Brassicanapus*. Plants were evaluated at 35 days postinoculation (dpi) by root dipping into respective spore solutions or water (Mock). (c) Representative plants (above) and overview pictures from a single experiment (below) are shown. (d) The diagram displays the number of plants with indicated symptoms relative to the total number of plants (*n*) assessed in two independent experiments. (e) *Verticillium* DNA in rapeseed hypocotyl was quantified at 35 dpi with respective spore solutions or water (Mock). *Verticillium*-specific ribosomal DNA was detected by quantitative PCR. Values relative to Vl43 are shown as percentages. (f) Reisolation of Vl43 and Vl32 from surface-sterilized stem slices of infected plants on potato dextrose agar with chloramphenicol after incubation at 25 °C for 7 days was possible. Less outgrowth was detected for Vl32 (red arrows)

(Depotter et al., 2016; Karapapa et al., 1997). So far, interspecific vegetative hyphal fusions of haploid *Verticillium* spp. have only been observed for auxotrophic mutants under selection (Hastie, 1973, 1989).

The adaptive evolution of fungal pathogens results in two-speed genomes with a conserved core and a more flexible pan-genome encoding genes required for infection (Dong et al., 2015), such as effectors supporting host colonization by immune response suppression or manipulation of the host's cell physiology as virulence factors (Faino et al., 2016; Gibriel et al., 2019; de Jonge et al., 2013; Kombrink et al., 2017; Selin et al., 2016; Stergiopoulos & de Wit, 2009). The organization of the pan-genome can have different forms. Some fungi carry entire lineage-specific (LS) chromosomes with pathogenicity-related genes (Galazka & Freitag, 2014).

Another nature of karyotype variation was observed in *Verticillium* spp., which display differences in length of chromosomes with rearranged sequence synteny (Faino et al., 2016; de Jonge et al., 2013; Shi-Kunne et al., 2018). A correlation between these chromosomal synteny interruptions, an enrichment of repetitive sequences, and the occurrence of LS regions, which are specific for single isolates or subgroups of strains or species, was observed in *V. dahliae* (de Jonge et al., 2013). Repetitive sequences contribute to centromere diversity and evolution in *Verticillium* (Seidl et al., 2020) and the relative number of genes for niche adaptation is enriched in LS regions compared to the core genome (Gibriel et al., 2019; de Jonge et al., 2013). A correlation of repeat-rich genomic regions and genes for niche adaptations was also observed in other plant pathogens, such as *Leptosphaeria maculans* (Rouxel et al., 2011).

Some *V. dahliae* LS regions were acquired through horizontal gene transfer from *Fusarium oxysporum* (Chen et al., 2018), from plants (de Jonge et al., 2012), or by transposon-mediated processes (Faino et al., 2016; Klosterman et al., 2011). LS regions show higher coding and noncoding sequence conservation compared to the core genome, possibly caused by differences in chromatin organization rather than by horizontal gene transfer (Depotter et al., 2019).

We compared the genomes of the virulent Vl43 (A1/D1) and the asymptomatic Vl32 (A1/D3) *V. longisporum* strains, which were both isolated from rapeseed in the same area in northern Germany (Zeise & von Tiedemann, 2002). It was evaluated whether the original hybridizations were the result of sexual mating or vegetative hyphal fusions. The contributions of the parental strains to the genetic setup of both hybrids were analysed. Intriguingly, deletion of an LS region, which was only present in pathogenic Vl43, did not reduce disease symptom induction, but instead increased the virulence of the pathogenic strain. This unexpected result supports that the corresponding genes, encoding presumed transcription factors, provide functions to reduce disease symptoms and restrict damage in the host plant.

2 | RESULTS

2.1 | Symptomatic and asymptomatic *V. longisporum* isolates Vl43 and Vl32 exhibit similar root colonization behaviour

V. longisporum Vl43 and Vl32 were isolated from rapeseed fields in the same area in Germany. Vl43 can be highly virulent, whereas plants infected with Vl32 did not show significant differences compared to water controls (Zeise & von Tiedemann, 2002). Each amphidiploid strain derived from a different hybridization event of haploid parental strains (Figure 1a). The infection and colonization phenotypes of both strains were compared (Figure 1). Fungal colonization of plant roots represents the first contact followed by penetration into the host. Comparison of Vl32 and Vl43 growth behaviour on *Arabidopsis thaliana* roots showed similar surface colonization (Figure 1b). Both strains formed hyphae growing along the interface of two plant cells. Thickening of hyphae suggested formation of invasion structures.

Only Vl43 induced visible disease symptoms in rapeseed (Figure 1c,d). At 35 days postinoculation (dpi) with Vl43 spores 27% of the plants had died. The remaining plants showed strong (30%), weak (27%), or no disease symptoms (17%), whereas all Vl32-treated plants appeared mock-like. Amounts of fungal rDNA in hypocotyls of surviving plants dropped more than 10-fold after Vl32 compared to Vl43 infection (Figure 1e). Both *V. longisporum* strains could be reisolated from stems of infected plants with strong fungal outgrowth of Vl43 and lesser growth of Vl32 (Figure 1f), suggesting that both isolates are able to colonize the plant's central cylinder, but Vl32 to a smaller extent.

It was examined whether the pathotype is connected to differences in the ex planta phenotypes of the strains. Four *V. longisporum* strains were tested on different media, including two strains of the lineages A1/D1 (Vl43, Vl145c) and A1/D3 (Vl32, Vl19), as well as *V. dahliae* (VdJR2, Vd39), *V. alfalfae* (Va2), and *V. nonalfalfae* (Va4) strains. All strains could grow on standard media and media with sorbitol or sodium dodecyl sulphate (SDS) as stressors (Figure S1). Individual strains displayed growth and melanization differences, but no clear correlation between phylogenetic lineage and phenotypes under these conditions. A1/D3 strains showed fluffier growth in comparison to A1/D1 strains on malt extract agar (Figure S2). Thick hyphal cables covered Vl32 and Vl19 colonies, but were rarely observed for A1/D1 strains. Scanning electron microscopy resolved these cables as bundles of single hyphae.

Overall, the ex planta phenotypes of isolates from symptomatic and asymptomatic *V. longisporum* lineages were similar with the exception that asymptomatic strains provide the potential for hyphal cable formation under specific conditions. Root surface colonization behaviour of Vl43 and Vl32 is similar, but further plant colonization is different and results in distinct pathogenic consequences for the host upon infection with Vl43.

2.2 | Vl32 and Vl43 carry same-sex mating-type genes derived from both parental lineages and form vegetative hyphal fusions

Whole-genome sequencing of *V. longisporum* Vl43 and Vl32 was performed to dissect the genetic basis for the different infection phenotypes. The hybrid *V. longisporum* genome is expected to contain twice the amount of DNA (c.66 Mb) compared to haploid *Verticillium* species (*V. dahliae:* c.36 Mb, Faino et al., 2015; *V. alfalfae:* c.30 Mb, Klosterman et al., 2011) and two copies for the majority of genomic loci (Clewes et al., 2008; Inderbitzin, Davis, et al., 2011; Tran et al., 2013). Previous *V. longisporum* sequencing data (VL1, VL2, VL20, VLB2) suggested a genome size of c.70 Mb (Depotter, Seidl, et al., 2017; Fogelqvist et al., 2018).

A high similarity between loci from the parental genomes makes the assembly of loci within their correct genomic environment challenging. Highly similar regions lead to fragmented assemblies, especially with short sequence reads. First Vl43 genome assemblies based on a combination of 454 and paired-read Illumina/Solexa data (Nowrousian et al., 2010) resulted in a total size of the expected c.70 Mb, but a high degree of fragmentation. Three libraries of long jumping distance (LJD) reads were sequenced to improve the assembly continuity (Table S1). Velvet assembly of the combined data (Zerbino & Birney, 2008) resulted in c.75 Mb with an N50 of 1,750 kb, but still a high number of assembly gaps, indicating great improvement of long-range continuity but internal regions of high similarity could not be resolved. A similar strategy based on 454 paired-end and LJD reads was used for the Vl32 genome assembly, resulting in c.69 Mb. While having a smaller N50 than the Vl43 assembly due to the use of only one LJD library, there are many fewer internal gaps, indicating fewer problems with highly

similar regions. Additionally, a draft genome assembly of pathogenic A1/D1 strain Vl145c was generated based on one Illumina/Solexa paired-end library (Tables S2 and S3).

The sexual pathway genes of Vl32 and Vl43 genomes were compared to address the hybridization origins between the two *Verticillium* lineages. Key functions include genes for potential receptors, pheromones, and mating-type. Two pheromone classes are involved in the sexual reproduction of filamentous ascomycetes: the *Saccharomyces cerevisiae* Pre-Pro-alpha factor-like peptide pheromone precursors and pheromones similar to yeast Mfa lipopeptide pheromone (Coppin et al., 2005; Kim & Borkovich, 2006; Mayrhofer et al., 2006; Pöggeler, 2011). The Vl43 genome includes two alleles encoding lipopeptide receptors (*PRE1-1*, *PRE1-2*) and peptide receptors (*PRE2-1*, *PRE2-2*), and two alleles encoding Ppg1-like peptide pheromone precursors (*PPG1-1*, *PPG1-2*, Figure S3a, sequences in Methods S1). The *Verticillium* lipopeptide pheromone differs from typical Ppg2 precursors in Sordariaceae. *Verticillium* Ppg2 resembles the hybrid-type pheromone precursors identified in the order Hypocreales (Schmoll et al., 2010), as it has the Ppg2-typical C-terminal CaaX motif, but it contains five repeats of a dodecapeptide sequence usually found in Ppg1 precursors (Figure S3b). Ppg2-like precursors of *Verticillium* lack a signal sequence and are, presumably, secreted by nonclassical STE6-mediated secretion (Figure S3c).

Typical mating-type (*MAT*) loci of heterothallic mating partners among filamentous ascomycetes consist of dissimilar *MAT1-1* and *MAT1-2* idiomorphs (Turgeon & Yoder, 2000). *MAT1-1* invariably contains the *MAT1-1-1* gene encoding an α-box domain protein. The *MAT1-2* locus carries *MAT1-2-1* encoding a protein with an HMG DNA-binding domain (Debuchy et al., 2010). Both mating-type loci can include additional genes: most Sordariomycetes *MAT1-1-2* loci encode a protein with a PPF (proline/proline/phenylalanine) domain, or *MAT1-1-3* encodes another HMG domain DNA-binding protein. Genomes of self-fertile (homothallic) filamentous ascomycetes contain either linked or unlinked genes indicative of both mating-types (Debuchy & Turgeon, 2006; Pöggeler, 2001).

V. longisporum isolates Vl43, Vl32, and Vl145c each contain two *MAT-1-1* alleles (Inderbitzin, Davis, et al., 2011). BLAST searches failed to detect *MAT1-2* homologs. The Vl43 genome assembly was not sufficiently complete to determine whether the arrangement of the mating-type genes of the *V. longisporum MAT1-1* idiomorphs is similar to the *MAT1-1* idiomorphs of *V. dahliae* (D-type) or *V. alfalfae* (A-type) because the mating-type idiomorph flanking gene *SLA2* was distributed on different scaffolds. PCR experiments using Vl43 DNA as template and the Vl32 assembly revealed that both mating-type loci are flanked by *SLA2* and *APN2*. The idiomorph sequences encode the mating-type genes *MAT1-1-1* and *MAT1-1-3* in a tail-to-tail orientation (Figure 2a, Table S4, and Sequences in Methods S1). Vl32 and Vl43 Mat1-1-1 and Mat1-1-3 proteins display high amino acid sequence identity when compared among the A- or D-types, but a reduced level of amino acid sequence identity when compared between the A- and D-type loci of the same strain. Mat1-1-1 and Mat1-1-3 proteins are more similar to *V. dahliae* than to *V. alfalfae* proteins. Mating-type

proteins of the Vl32 *MAT1-D* locus are 100% identical to *V. dahliae* Mat1-1-1 and Mat1-1-3 (Tables S5 and S6, and Figure 2b). The main sequence differences between *A* and *D* loci are in the intergenic region of *MAT1-1-3* and *APN2* genes. The *MAT1-D* locus contains a short 295 bp sequence with high sequence similarity to hypothetical open reading frames (ORFs) located in the *MAT*-flanking regions of *V. dahliae* (VDAG_06529) and *V. alfalfae* (VDBG_03574.1) (Figure 2b). In contrast, the intergenic *MAT1-1-3/APN2* region of Vl43 and Vl32 *MAT-A* loci contains the entire coding region of VDAG_06529.

V. longisporum Vl43 and Vl32 carry two *MAT1-1* alleles, one from each parent, and miss an opposite *MAT1-2* idiomorph. One *MAT1-1* locus present in both *V. longisporum* isolates contains an ORF homologous to VDAG_06529 and VDBG_03574, which is not localized adjacent to the mating-type genes in *V. dahliae* or *V. alfalfae*. This might reflect the genomic situation of the yet unidentified parental strain A1. The finding that both mating alleles in Vl43 and Vl32 belong to the same idiomorph suggests that Vl43 and Vl32 are presumably not derived from a mating event or, if hybridization is the result of mating, a homogenization of the initial loci must have happened.

Scanning electron microscopy explored the possibility of same-sex hybridization events. Individual hyphae of *V. longisporum* can be interconnected by anastomoses (Figure 2c). This was frequently observed within the hyphal cables of asymptomatic (Vl32, Vl19), but rarely in pathogenic (Vl43, Vl145c) isolates, most often in close contact with the agar surface. It is yet unclear whether same-sex hybridization was the origin of *V. longisporum* because anastomoses could only be observed for hybrid and not for haploid putative parental strains.

2.3 | The two *V. longisporum* genomes display minimal gene variations but different numbers of massively rearranged chromosomes

The two parental origins of Vl32 and Vl43 hybrid genomes were visualized by plotting the relative identity between either of the two *V. longisporum* isolates against *V. dahliae* long DNA contigs (Figure 3a). Both plots display two similarity peaks: one close to 100% identity, resembling the D1/D3 component, the other 94% identity of A1 origin. Plotting of *V. alfalfae* against *V. dahliae* sequences visualizes that *V. dahliae* is approximately as related to *V. alfalfae* as to A1. As control, the plotting of two *V. dahliae* strains, VdLs.17 (Klosterman et al., 2011) and VdJR2 (de Jonge et al., 2012; Faino et al., 2015; Fradin et al., 2009), resulted in almost 100% identity.

V. longisporum gene gain or loss after hybridization was addressed by comparing predicted ORFs against the scaffolds of relative *Verticillium* spp. using a BLAST-based clustering approach. The number of genes unique to each *V. longisporum* genome was below 1% (Figure 3b). Analysis of the putative *V. longisporum* strain-specific genes demonstrated that the respective regions are very short (1 to 11 genes).

V. dahliae and *V. alfalfae* genomes consist of seven or eight chromosomes, as shown by restriction fragment length polymorphism

(a)

(b)

(c)
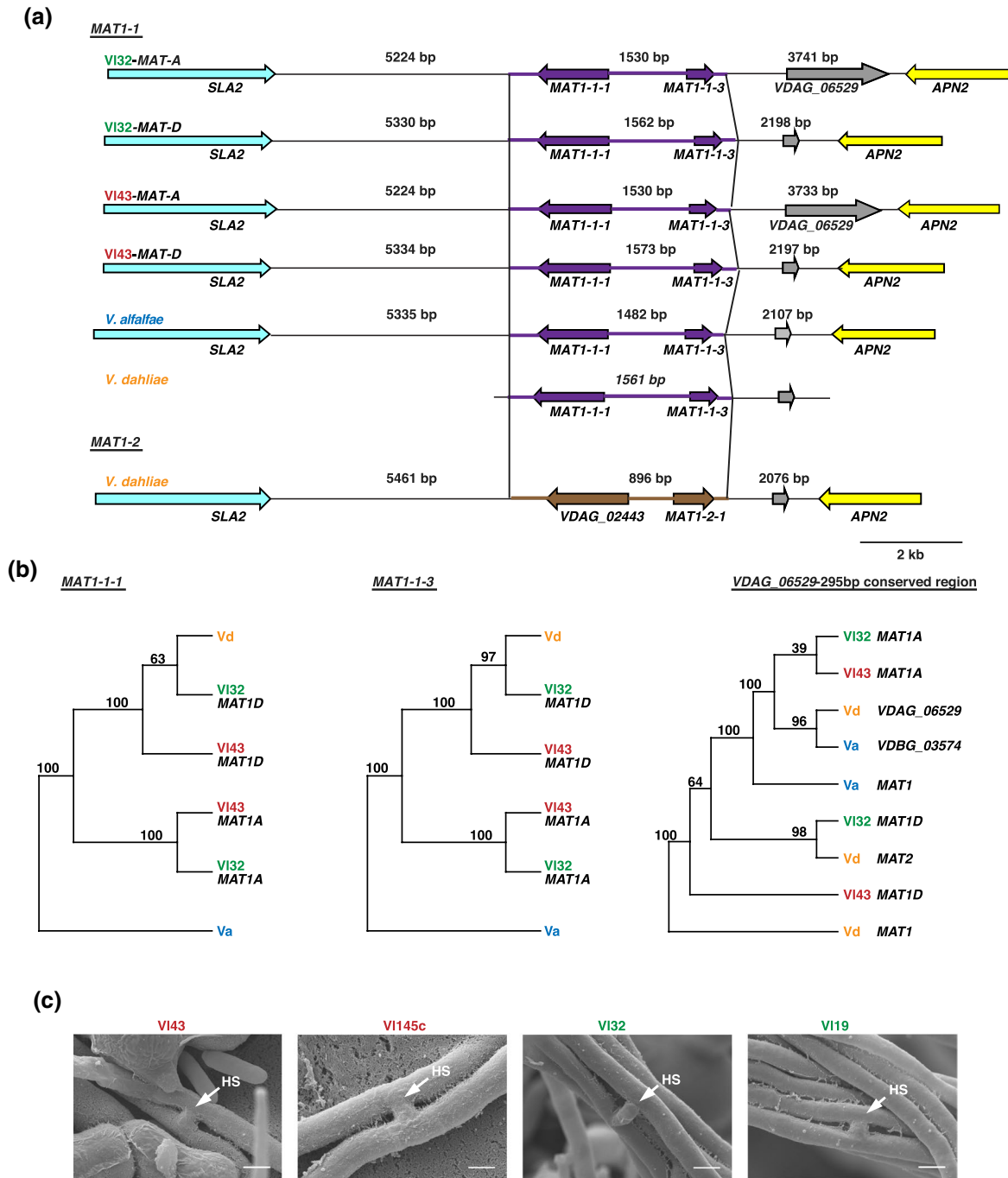
FIGURE 2 *Verticillium longisporum* Vl32 and Vl43 harbour same-sex mating-type genes originating from both parental lineages. (a) Comparison of *V. longisporum* Vl32 and Vl43 *MAT1-1* and *V. dahliae* (Vd)/*V. alfalfae* (Va) mating-type loci with flanking regions. Lines: intergenic regions; purple arrows:*MAT1-1*-specific regions; brown arrows:*MAT1-2*-specific regions; vertical line: border of the idiomorph sequence. Sequences from Va *MAT1-1* and Vd *MAT1-2* loci from Ensembl Fungi (Kersey et al., 2018) and Vd *MAT1-1* (AB505215.1) were used. (b) Phylogenetic tree of *MAT1-1-1*, *MAT1-1-3*, and a conserved 295-bp region of *VDAG_06529* homologs. Consensus trees based on nucleotide sequences were calculated with PHYLIP program NEIGHBOR. Percentages based on 1,000 replications of the neighbour-joining procedure are shown. (c) Scanning electron micrographs of hyphal fusion sites (HS) from *V. longisporum* strains of lineage A1/D1 (Vl43, Vl145c) or A1/D3 (Vl32, Vl19) cultivated on malt extract agar for 3 days are shown (scale bar: 2 μm)

and optical mapping (Faino et al., 2015; Klosterman et al., 2011; Pantou & Typas, 2005). Optical mapping of both genomes revealed that the Vl32 genome is distributed among 16 chromosomes, which fits with the genome duplication (Figure 4a). In contrast, the Vl43 genome consists of only 15 chromosomes, one less than in Vl32.

Comparing chromosome sizes between Vl43 and Vl32 we concluded that the difference is not only an additional chromosome for Vl32, but a difference in length for all chromosomes.
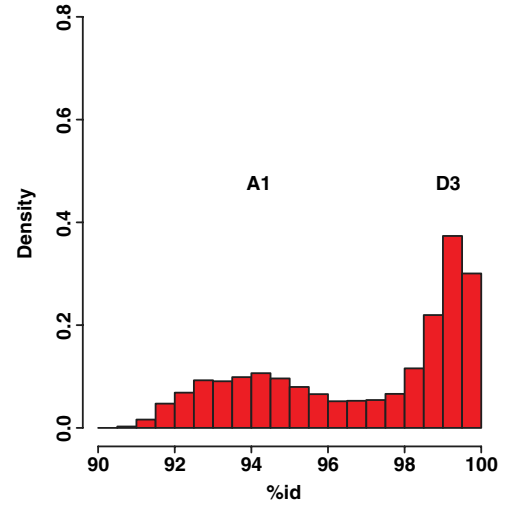
Analysis of syntenic regions between Vl43, Vl32, and VdLs.17 showed a much larger overlap between Vl32 and the *V. dahliae* isolate

(a)

V. dahliae vs. VI43
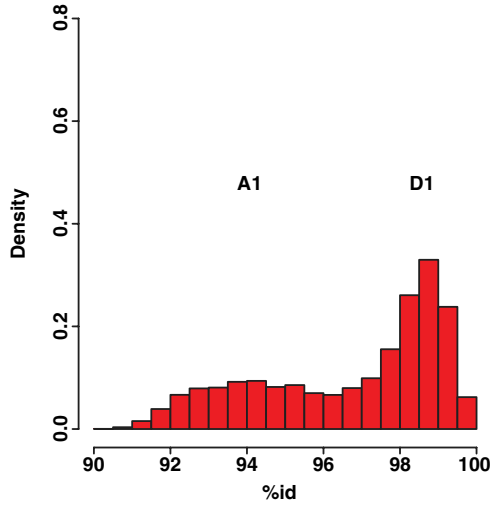
V. dahliae vs. VI32

V. dahliae vs. V. alfalfae
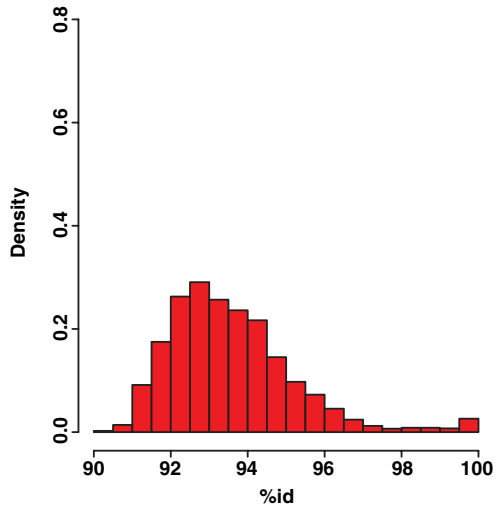
V. dahliae vs. V. dahliae
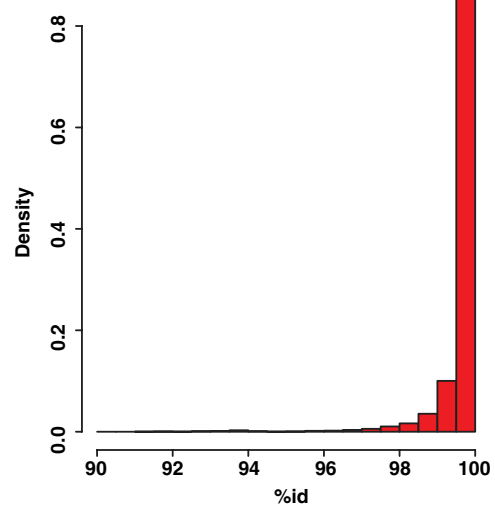
(b)

Number of genes specific for

VI43 (21,176 [a])	VI32 (19,313 [a])	V. dahliae (10,535 [b])	V. alfalfae (10,221 [b])	versus
-	73	233	144	VI43
149	-	227	180	VI32
49	87	216	146	VI145c
-	69	204	134	VI43 + VI145c
-	-	130	121	VI43 + VI145c + VI32
455	386	-	296	V. dahliae
863	676	585	-	V. alfalfae

FIGURE 3 *Verticillium longisporum* strains are two-parent hybrids. (a) In Vl43 and Vl32 genomes, two identity groups are bimodally resembled in the distribution of relative identity (%id) of long genomic contig (>1,000 kb) alignments. Contigs of Vl43, Vl32, VaMs.102, and VdJR2 were compared to the VdLs.17 genome using Exonerate software (Slater & Birney, 2005). (b) Strain-specific genes in *Verticilllium* genomes. Numbers given in the top row represent the total amount of predicted open reading frames (ORFs). VdLs.17 and VaMs.102 were used as *V. dahliae* and *V. alfalfae* reference genomes, respectively (Ensembl Fungi; Kersey et al., 2018). Total numbers of predicted ORFs per genome according to Augustus prediction[a] and Klosterman et al. (2011)[b] are given

(14 Mb) than between Vl43 and VdLs.17 (<1 Mb) (Figure 4b). Vl32 and Vl43 shared approximately 26 Mb syntenic regions, whereas only approximately 1 Mb are in synteny between all strains. Collinear blocks of the 15 Vl43 and the 16 Vl32 chromosomes were visualized (Figure 4c). The high number of lines connecting the syntenic blocks of the respective chromosomes indicates massive syntenic rearrangements. These can correlate to repetitive sequences (Depotter et al., 2016; Faino et al., 2016; Möller & Stukenbrock, 2017; Seidl & Thomma, 2017). In addition, 10% and 12% of the Vl32 and Vl43 genomes consist of repeats compared to 4% of *V. dahliae* (Table S7). The de novo analysis found repetitive sequences of *V. alfalfae* and *V. dahliae* do not give much more repeat masking than with the general RepeatMasker libraries. In contrast, in the *V. longisporum* strains, de novo analysis found repeats mask a much higher percentage of the genome. Similar to the ascomycete *Pyronema confluens* (Traeger et al., 2013), this indicates a more recent expansion of *V. longisporum* repetitive sequences (Figure S4).

Our comparisons suggest minimal gene gain or loss in the hybrid genomes compared to *V. dahliae* and *V. alfalfae*. The plant colonizer Vl32 has 16 chromosomes, whereas the symptom inducer Vl43 has only 15. *V. longisporum* genomes are massively rearranged and enriched with repetitive sequences.

2.4 | A 20 kb genomic region absent in asymptomatic *V. longisporum* Vl32 reduces disease symptom induction in *Brassica napus* by virulent Vl43

Comparison of asymptomatic *V. longisporum* Vl32 to pathogenic Vl43 revealed few specific genes within the two amphidiploid genomes. Genomic rearrangements and evolution of LS regions correlate in haploid *Verticillium* spp. (Chen et al., 2018; de Jonge et al., 2013; Gibriel et al., 2019; Klosterman et al., 2011). Four LS regions (LS1–4) of about 300 kb in the genome of *V. dahliae* VdLs.17 (Faino et al., 2016) share no synteny to the *V. alfalfae* VaMs.102 genome (Klosterman et al., 2011). These four VdLs.17 LS regions with enriched repetitive sequences might increase genetic flexibility for *V. dahliae* to adapt to different host niches. A bioinformatic search for VdLs.17 orthologs present in the Vl43 genome was performed to identify candidates important for this *V. longisporum* pathotype. Small LS1, LS3, and LS4 subregions were conserved in synteny in pathogenic A1/D1 *V. longisporum* isolates Vl43 and Vl145c, as well as in haploid *V. dahliae* JR2 (Figure 5a). This synteny was disrupted in the asymptomatic A1/D3 isolate Vl32. The small *Vl43LS20kb* subregion of approximately 20 kb was present in Vl43, but absent from the Vl32 genome and also from the genome of haploid *V. alfalfae* VaMs.102. The absence of this region in Vl32 was verified by

PCR analyses targeting seven genes (LS region gene = *LSG1–LSG7*, Figure 5b, Table S8) predicted for the *Vl43LS20kb* region (gene annotations according to *V. dahliae* JR2 in Ensembl Fungi; Faino et al., 2015; de Jonge et al., 2012; Kersey et al., 2018). *LSG3* and *LSG6* code for potential transcription factors. All *LSGs* are present in single copy in *V. longisporum* Vl43 or Vl145c. Instead, two copies for *LSG1*, one not yet annotated (in the *Vl43LS20kb* homologous region between *VDAG_JR2_Chr2g10300a* and *VDAG_JR2_Chr2g10310a*) and a second copy on chromosome 5 (*VDAG_JR2_Chr5g10950a*) are present in the haploid *V. dahliae* JR2 genome.

Vl43 deletion strains were examined to determine whether the pathogen-specific *Vl43LS20kb* region encodes pathogenicity factors. The *Vl43LS20kb* region was divided into two subregions (*LSI*: c.11.5 kb with *LSG1–LSG5*; *LSII*: c.8.5 kb with *LSG6–LSG7*). *LSI*, *LSII*, or the total *LS* region were deleted in Vl43 (Figures 5a and S5a,b). Vl43Δ*LSI*, Vl43Δ*LSII*, and Vl43Δ*LS* spot inoculated onto different media with or without stress-inducing agents appeared like the wild type ex planta (Figures 5c and S5c). In rapeseed infection experiments, the Vl43Δ*LSI*, Vl43Δ*LSII*, and Vl43Δ*LS* strains induced more severe disease symptoms after 35 days compared to plants inoculated with wild-type Vl43 spores (Figure 5d,e). Also, 27% of the plants inoculated with the wild-type Vl43 died from fungal infection, whereas there were 70% dead plants for Vl43Δ*LSI*, 83% for Vl43Δ*LSII*, and 89% for Vl43Δ*LS*. The small amount of surviving plants at the end of the experiment did not allow a quantification of fungal DNA.

The *Vl43LS20kb* region is in the same syntenic arrangement in the pathogenic A1/D1 *V. longisporum* isolate Vl145c and in *V. dahliae* JR2. The *Vl43LS20kb* homologous region in *V. dahliae* JR2 was deleted (Figure S6a,b). The ex planta phenotypes of *V. dahliae* JR2Δ*LS* spot inoculated onto different media revealed that the *LS* region is dispensable for vegetative growth of *V. dahliae*, as found for *V. longisporum* Vl43 (Figure S6c). Tomato plants were inoculated with conidiospores from two independent *V. dahliae* JR2Δ*LS* transformants. Severity of disease symptoms was unaltered for both wild-type- and JR2Δ*LS*-treated plants after 21 days (Figure S6d,e). Hypocotyl cross-sections of *V. dahliae* JR2Δ*LS*-infected plants showed wild-type-like discolouration, indicating no alterations in plant defence responses. The potential of *V. dahliae* JR2Δ*LS* to induce disease symptoms in rapeseed was tested and revealed wild-type-like absence of disease symptoms in treated plants (Figure S7).

In summary, the *Vl43LS20kb* region of pathogenic *V. longisporum* Vl43 fulfils an unexpected function, which differs to the corresponding *V. dahliae* JR2 region. In contrast to the prediction that *Vl43LS20kb*, which is absent in the asymptomatic isolate Vl32, contributes to virulence, this region even tames the pathogenic features of Vl43 and reduces disease symptom severity induced in rapeseed.
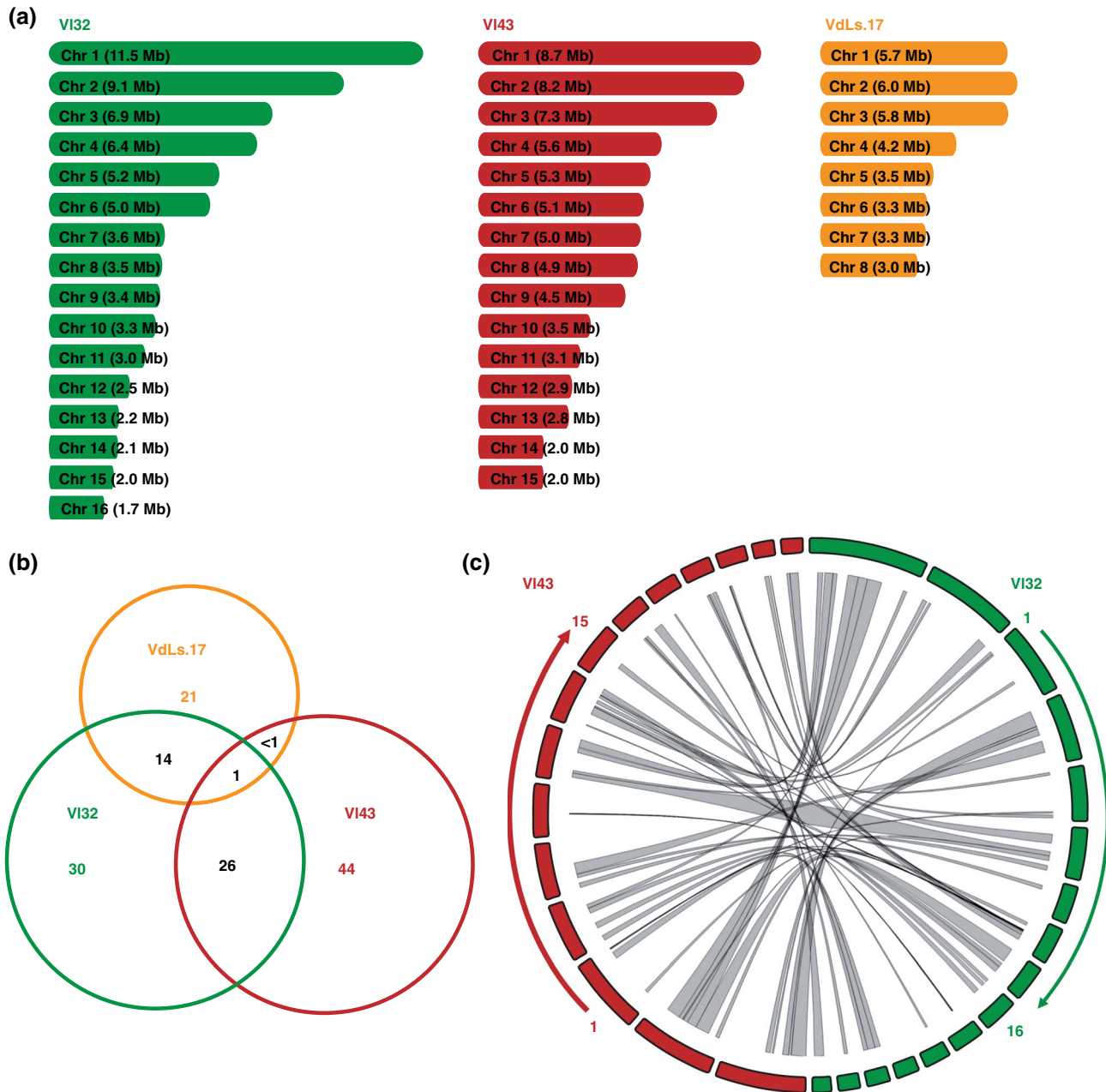
FIGURE 4 Genome rearrangements between *Verticillium longisporum* Vl43 and Vl32. Mapping is based on restriction patterns determined by optical mapping (OptGen). (a) Illustration of Vl43 and Vl32 chromosome numbers and sizes compared to *V. dahliae* VdLs.17 (de Jonge et al., 2013). (b) The Venn diagram illustrates the length (Mb) of overlapping and unique syntenic regions in Vl43, Vl32, and VdLs.17 genomes. Genome synteny is partially conserved between Vl32 and VdLs.17 and between Vl32 and Vl43. Synteny overlap between Vl43 and VdLs.17 is very low. (c) Chromosome synteny comparison in a Circos diagram reveals a high level of genomic rearrangements by illustration of collinear blocks between 15 Vl43 (red) and 16 Vl32 (green) chromosomes.

This suggests a trade-off strategy in the interaction between a fungus and its host plant, resulting in evolutionary advantages.
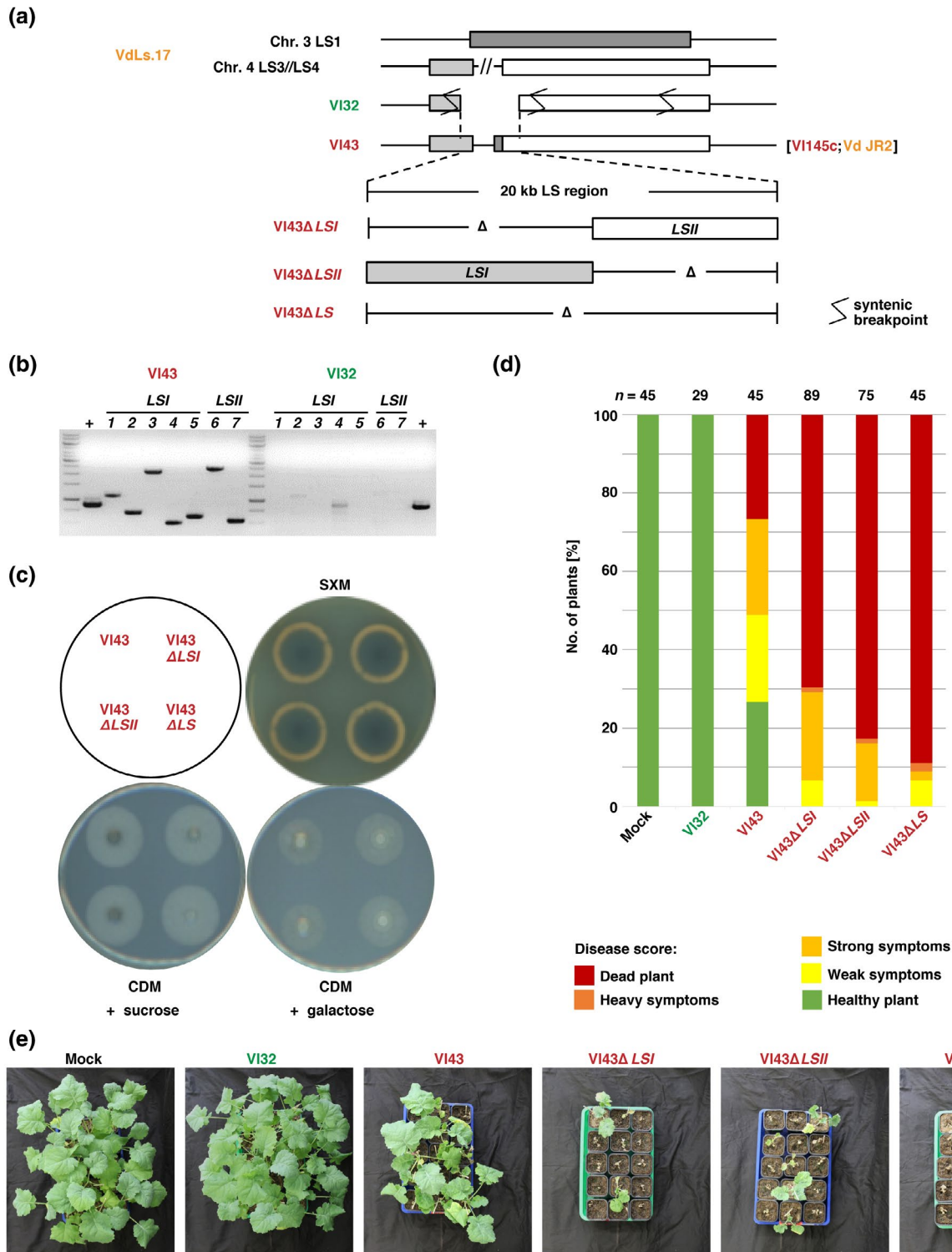
3 | DISCUSSION

V. longisporum Vl43 and Vl32 are two representatives of interspecies lineages deriving from distinct hybridizations (Inderbitzin, Davis, et al., 2011). Vl43 of lineage A1/D1 causes severe symptoms in rapeseed plants, whereas Vl32 of the A1/D3 lineage colonizes plants asymptomatically (Depotter, Rodriguez-Moreno, et al., 2017; Zeise & von Tiedemann, 2002). Vl43 is also able to cause symptoms in *Arabidopsis thaliana* (Reusche et al., 2012). Both strains colonize roots similarly, but fungal biomass reisolated from infected tissue is higher for Vl43 than for Vl32. Successful root penetration is performed by both, but they differ in subsequent steps of host plant colonization

resulting in higher amounts of fungal Vl43 DNA in hypocotyls of infected plants. This is similar in nonpathogenic compared to pathogenic *F. oxysporum* (Validov et al., 2011). *Verticillium* spp. colonize the host plant's vascular system, which is nutrient-poor and imbalanced in amino acids (Singh et al., 2010). *V. longisporum* recognizes this environment and responds with a highly specific secretion pattern, including pathogenicity-related effectors (Leonard et al., 2020).

The genomes of *V. longisporum* Vl43 and Vl32 with different pathotypes revealed high gene level conservation, but different chromosome numbers and sizes combined with increases in repetitive sequences. Vl32 and Vl43 carry genes for potential pheromones and their receptors as well as two *MAT1-1* same-sex mating-type genes derived from both parental strains, but no *MAT1-2*. One of these parental alleles contains an additional complete ORF of unknown

(a)

(b)

(c)

(d)

Disease score:
- Dead plant (red)
- Heavy symptoms (orange)
- Strong symptoms (orange/yellow)
- Weak symptoms (yellow)
- Healthy plant (green)

(e) Mock | Vl32 | Vl43 | Vl43ΔLSI | Vl43ΔLSII | Vl43ΔLS

FIGURE 5 The *Vl43LS20kb* region of *Verticillium longisporum* Vl43 reduces severity of *Brassica napus* disease symptoms. (a) Arrangement of the 20 kb lineage-specific (LS) region and adjacent regions in pathogenic (red) and asymptomatic (green) *V. longisporum* strains compared to *V. dahliae* VdLs.17 LS regions LS1 (dark grey), 3 (light grey), and 4 (white). Parts of VdLs.17 LS1 located on chromosome 3 as well as parts of LS3 and LS4 regions located on chromosome 4 are in synteny in the genomes of Vl43, Vl145c, and VdJR2, but disrupted in the Vl32 genome at indicated breakpoints. The 20 kb *LS* region is absent from the Vl32 genome. The *Vl43LS20kb* region was subdivided into *LSI* and *LSII*. Vl43 deletion strains lack *LSI*, *LSII*, or the total 20 kb *LS* region. (b) Absence of the *Vl43LS20kb* region in the Vl32 genome was verified. Seven genes (*LSG1–LSG7*) were amplified from DNA of Vl43, but not of Vl32. Histone *H2A* served as positive control (+). (c) Ex planta phenotypes of Vl43Δ*LSI*, Vl43Δ*LSII*, Vl43Δ*LS* strains on simulated xylem fluid medium (SXM; bottom view) and Czapek Dox medium (CDM) with sucrose or galactose (top view) for 14 days at 25 °C show no significant alterations compared to Vl43. (d) and (e) *V. longisporum* Vl43Δ*LSI*, Vl43Δ*LSII*, and Vl43Δ*LS* induce more severe disease symptoms in *B. napus* compared to wild-type Vl43 and asymptomatic Vl32 35 days postinoculation by root dipping into respective spore solutions or water (Mock). (d) The diagram displays the number of plants with indicated symptoms relative to the total number of plants (*n*) from three experiments with two independent Vl43Δ*LSI*, Vl43Δ*LSII* transformants and one Vl43Δ*LS* transformant. (e) Representative overview pictures of plants from a single experiment

function, which might reflect the genomic situation of the yet unknown A1 strain. If hybridization is based on mating, the initial locus must have been homogenized as found for the rDNA genes (Tran et al., 2013). Because *MAT1-1* loci are rare in haploid *Verticillium* spp., hyphal anastomosis and nuclear fusions are more likely. However, only self-anastomosis of Vl43 and Vl32 was achieved, which could not be verified with haploid species.

The increased chromosome number in asymptomatic Vl32 was not due to an accessory chromosome comprising species-specific genes but arose from rearranged genomic regions. Similarly, frequent synteny breakpoints caused by genomic rearrangements occur in all *Verticillium* spp. (Shi-Kunne et al., 2018). In eukaryotes, chromosomal reshuffling is often linked to phenotypical alterations and gene gains or losses affect fitness levels in different niches (Coghlan et al., 2006; Dong et al., 2015; Plissonneau et al., 2018; Tang & Amon, 2013).

Genomic rearrangements and the evolution of LS regions correlate in haploid *V. dahliae* (Chen et al., 2018; Faino et al., 2016; Gibriel et al., 2019; de Jonge et al., 2013; Klosterman et al., 2011). In *V. dahliae* VdLs.17, host range expansion and niche adaptation compared to *V. alfalfae* might be mediated by LS1–4 regions (Klosterman et al., 2011). Different *V. dahliae* isolates showed increased numbers of effector genes in LS regions when compared to the core genome, although the number of effectors was small relative to the total number of genes encoded in the LS region (Gibriel et al., 2019; de Jonge et al., 2013). Most studies have identified LS regions by comparison of *V. dahliae* isolates. *V. dahliae* and *V. alfalfae* genomes comprise the same relative numbers of genes for secreted proteins in the core and LS regions (Klosterman et al., 2011). Within the flexible regions, transposable elements, gene duplications as well as genes linked to pathotypes, regulation of transcription, signalling, degradation of organic material, and iron or lipid metabolic processes can be found (Klosterman et al., 2011). Some of these might be connected to niche adaptation. LS regions of *V. dahliae* could represent a unique chromatin state with regard to transposable elements, methylation marks, and DNA accessibility when compared to the core genome (Cook et al., 2020).

We discovered that a genomic LS region, originating from the *V. dahliae*-related parental lineage D1, is present in pathogenic isolates Vl43 and Vl145c, but absent in the genome of the asymptomatic *V. longisporum* isolate Vl32. The *Vl43LS20kb* homologous region

of *V. dahliae* JR2 seems not to play a role in pathogenicity towards tomato or rapeseed plants. Characterization of the *Vl43LS20kb* region in *V. longisporum* Vl43 revealed that this region does not contain virulence factors that explain the difference of this pathogenic strain to the asymptomatic Vl32. In contrast, this region has a negative impact on disease symptom severity induced in *V. longisporum* Vl43-colonized rapeseed plants and tames the fungus in its relationship with the plant. The virulence-suppressing function of the *Vl43LS20kb* region keeps Vl43 at an intermediate virulence level, which might be beneficial for fungal accumulation within the host plant. It is still unknown whether the genes of this region also affect other *Verticillium* species. The damage response network has been developed as a concept for describing microbial–host interactions ranging from commensalism to colonization to disease. These interactions differ in the degree of damage encountered by the host, which can be caused by the host response, by the microbe, or by the combination of both and make it difficult even within the same species to define what is a host and what is a pathogen (Casadevall & Pirofski, 2014, 2018). In line with this, isolates of the same *V. longisporum* lineage differ in pathogenicity on different host plants (Novakazi et al., 2015). These observations support the idea that different *Verticillium* strains of the same species display a high degree of specialization and adaptation to specific hosts depending on a variety of parameters. The acquisition of new genes might contribute to this specialization but, additionally, existing responses to the environment might be differentially regulated. The ex planta phenotypes of *LS* deletion strains resembled the wild-type Vl43, suggesting that the induction of more severe symptoms in planta and the detrimental impact on the host is not connected to increased fungal growth or propagation.

Gene losses due to chromosomal rearrangements can play significant roles in niche adaptation in various plant-colonizing fungi (Hartmann et al., 2017; Plissonneau et al., 2018; Sharma et al., 2014). Deletion of the *Vl43LS20kb* region might result in misregulation of virulence factors or virulence-attenuating factors. Consistently, the pathogenic isolate is less tolerable for rapeseed plants when the *Vl43LS20kb* region is absent. These virulence or virulence-attenuating factors might not be present or expressed in the asymptomatic colonizer Vl32.

Asymptomatic or less virulent isolates might contain pathogenicity-related genes in their genomes that are active or inactive depending on differences in the control of various transcriptional

networks. The *Vl43LS20kb* region identified in the pathogenic Vl43 strain encodes two potential transcription factors. Both proteins are candidates for the regulation of virulence-associated genes. Transcriptional control of virulence-related genes has been observed during analysis of related fungal species with different pathotypes. Transcriptome analyses of *V. dahliae* isolates with different potential to induce disease symptoms in cotton revealed decreased transcript levels of genes for virulence-connected proteins. Potential transcription factors for pathogenicity-related gene control were enriched in the less virulent strain (Jin et al., 2019). *V. dahliae* Vta2, Vta3, Som1, and Sge1 transcription factors control pathogenicity and development (Bui et al., 2019; Santhanam & Thomma, 2013; Tran et al., 2014). Transcription factors with negative impact on virulence include the pH-dependent regulator PacC of *F. oxysporum* for repression of low pH-expressed genes. PacC mutants are more virulent on tomato plants (Caracuel et al., 2003). *Alternaria brassicicola* Amr1 represses hydrolytic enzyme transcription (Cho et al., 2012). In other plant pathogens Amr1 regulates melanin biosynthesis, which is not necessarily linked to virulence in *V. dahliae* (Harting et al., 2020; Wang et al., 2018).

To our knowledge, this is the first study describing an LS region that decreases the virulence of a pathogenic *Verticillium* isolate. The asymptomatic isolate Vl32 lacks this region. Besides the addition or loss of genes coding for pathogenicity factors, regions required for pathogenicity attenuation contribute to the formation of different pathotypes. These regions and the encoded functions may help closely related strains to control the degree of damage in a specific host and in a defined environmental context.

4 | EXPERIMENTAL PROCEDURES

Strains, primers, and plasmids are listed in Tables S10–S12. Cultivation conditions, strain constructions, plant experiments, and phylogenetic analyses are described in Methods S1. Fungal transformants were verified by Southern hybridization (Bui et al., 2019).

4.1 | DNA preparation and sequencing strategy

Fungal genomic DNA for Southern hybridization and whole-genome sequencing was isolated from mycelium grown in potato dextrose broth (Carl Roth) for 1 week at 25 °C. Mycelium was harvested with Miracloth (Calbiochem Merck), rinsed, tissue-dried, ground in liquid nitrogen, and genomic DNA was isolated (Kolar et al., 1988). A single-stranded DNA shotgun library (ssDNA library) was generated from approximately 5 μg genomic DNA. DNA was fragmented by nebulization for 30 s at 1 bar. Further steps were done according to the Roche protocol. Sequencing was done using the Genome Sequencer FLX system (Roche Applied Science). Illumina sequencing was performed by GATC Services (Eurofins Genomics) using the Genome Analyzer SN365-Hi-Seq2000 with the TruSeq SBS v. 5 kit (Illumina). Sequenced libraries were processed using a sequence length cut-off

of 30 bp for paired sequences as well as for shotgun sequences. The input data for the assemblies can be found in Table S1.

4.2 | Assembly and *k*-mer frequency analysis

Before assembly, 454 and Illumina raw data were trimmed with custom-made Perl scripts as described (Nowrousian et al., 2012; Teichert et al., 2012). Details on assembly generation can be found in Methods S1. *k*-mer frequencies were analysed as described previously (Potato Genome Sequencing Consortium, 2011; Traeger et al., 2013) (Table S9 and Figure S8). Illumina/Solexa read pairs from paired-end libraries after trimming were used for the analysis.

4.3 | Genome annotation

Structures of Vl43 and Vl32 protein-coding genes were annotated with AUGUSTUS (Stanke et al., 2008), which was trained for *V. longisporum*. Learned parameters and evidence from the repeat masked genomes, homolog proteins, RNA-Seq, and EST data, and peptide sequences were used for gene prediction in the two genome assemblies (see Methods S1). In Vl43 and Vl32 genome assemblies 21,176 and 19,313 protein-coding genes were found, respectively. The median number of coding exons per gene was three for both genomes. The number of strain-specific genes was determined by a BLAST-based clustering approach using the predicted ORFs of *V. longisporum* Vl32 and Vl43, *V. dahliae* VdLs.17, and *V. alfalfae* VaMs.102 (Ensembl Fungi, http://fungi.ensembl.org/; Kersey et al., 2018).

4.4 | Repeat content analyses

Transposable elements and other repeats were analysed with RepeatMasker (A.F.A. Smit, R. Hubley, P. Green; RepeatMasker Open; www.repeatmasker.org) based on the RepbaseUpdate library (Jurka et al., 2005) and a library of de novo-identified repeat consensus sequences that was generated by RepeatModeler (A.F.A. Smit, R. Hubley; RepeatModeler Open; www.repeatmasker.org). Details can be found in Methods S1.

4.5 | Whole-genome comparison and visualization of collinear blocks

Optical maps of Vl32 and Vl43 were generated and visualized by OpGen using the Mapsolver software (OpGen).

4.6 | Sequence analyses

As reference genomes the following sequences in the Ensembl Fungi genome database (Kersey et al., 2018) were used: *V. dahliae* VdLs.17

(Klosterman et al., 2011) and JR2 (de Jonge et al., 2012), *V. alfalfae* VaMs.102 (Klosterman et al., 2011).

Conserved domains were predicted with the InterPro webpage (http://www.ebi.ac.uk/interpro/; Mitchell et al., 2019). Protein localization was analysed using DeepLoc 1.0 (http://www.cbs.dtu.dk/services/DeepLoc/; Almagro Armenteros et al., 2017). Nuclear localization sites were predicted by cNLS-Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi; Kosugi et al., 2009).

4.7 | Presence/absence verification of LS regions and analyses of *MAT* loci

The LS region and the Vl43 *MAT* locus were amplified by PCR from genomic DNA. For details see Methods S1.

4.8 | Light and electron microscopy

A binocular microscope (Olympus) with cellSens dimension software (v. 1.4; Olympus) was used for light microscopy of colonies, hyphae, and stem tissue. For details on electron microscopy see Methods S1.

DATA AVAILABILITY STATEMENT

The genome data are deposited under the BioProject accession numbers PRJNA643983, PRJNA643984, and PRJNA643985. Many predicted transcripts did not pass the filters of NCBI during genome submission. Thus, the number of originally predicted transcripts (used for this study) and the number of submitted transcripts differs. We therefore provide genomes and the full set of predicted transcripts at http://bioinf.uni-greifswald.de/bioinf/katharina/verticillium_full_data

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SUPPORTING INFORMATION

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