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FUNGAL DISEASES

Identification of the chromosome region responsible for pathogenicity of *Verticillium dahliae* on tomato using genetic recombination through protoplast fusion

Toshiyuki Usami[1] · Naoko Iida[1] · Keisuke Nakao[1] · Ayame Hamano[1] · Nana Suzuki[1] · Yusuke Ohmura[1] · Yoshimi Komiya[1]

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Abstract

The host range of *Verticillium dahliae*, causal agent of verticillium wilt in various dicot plants, differs among strains, but the mechanism responsible for the host-specific pathogenicity of the strains remains unclear. In this study, protoplast fusion of a tomato-pathogenic and a nonpathogenic strain of *V. dahliae* was used for genetic recombination of the isolates to localize the fungal genomic region involved in the pathogenicity on tomato. Twenty fusion strains resistant to two antibiotics, hygromycin B and geneticin, were obtained by protoplast fusion between two parental strains resistant to one of these antibiotics. Genomic Southern hybridization probed with telomere sequences revealed that these fusion strains were haploid and inherited chromosomes from both parental strains. Eight fusion strains were pathogenic on tomato. In PCR analysis of the fusion strains using DNA markers specific to a parental strain TV103, two DNA markers (T12 and VDA787) were amplified only in strains pathogenic on tomato. The genomic region mapped around these two DNA markers for a parental strain pathogenic on tomato was similar to that on the map for chromosome 3 of a reference strain (JR2). The analysis of 35 fusion strains with additional DNA markers revealed that one of the markers was completely accorded with pathogenicity of the strains on tomato. Therefore, the genomic region around this DNA marker is possibly involved in pathogenicity of *V. dahliae* on tomato.

Keywords Verticillium wilt · *Solanum lycopersicum* · Parasexual recombination · Protoplast fusion

Introduction

The soilborne plant pathogenic fungus *Verticillium dahliae* causes vascular wilt disease on many species of dicotyledenous plants. A single strain may typically be capable of invading multiple plant species, with the host range differing among strains (Pegg and Brady 2002). *V. dahliae* is known to be an asexual fungus (Usami et al. 2009b). However, genetic recombination via a parasexual cycle has been reported (O'Garro and Clarkson 1988, 1992; Typas and Heale 1978). Moreover, the host range of *V. dahliae* (Usami and Amemiya 2005) and other *Verticillium* species (McGeary and Hastie 1982) can vary following genetic recombination between strains, indicating that a gene(s) in the genome of those fungi controls specific pathogenicity for host plant species. The host range of each strain is probably determined by a combination of the genes, and genetic recombination has been shown not only in vitro but also *in planta* (Clarkson and Heale 1985; Wheeler and Johnson 2019). Such genetic recombination will lead to the appearance of strains having novel host ranges in the field.

Various genetic factors involved in virulence of *V. dahliae* have been identified in recent years. Chen et al. (2018) compared genomic sequences among *V. dahliae* strains and identified genes contributing specifically to virulence on cotton; however, these genes did not confer virulence on tomato and lettuce. On the other hand, de Jonge et al. (2012) reported that the gene *VdAve1* is a virulence factor in tomato. *VdAve1* was identified to encode an expancin- or natriuretic

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✉ Toshiyuki Usami
usami@faculty.chiba-u.jp

[1] Graduate School of Horticulture, Chiba University, 648 Matsudo, Matsudo-city, Chiba 271-8510, Japan

peptide-like product, which works as an avirulence factor to activate *Ve1*-mediated resistance in tomato (de Jonge et al. 2012). Race 2 strains of *V. dahliae* lack *VdAve1* and can overcome *Ve1*-mediated resistance (de Jonge et al. 2012). Race 2 is less virulent than race 1 (carrying *VdAve1*), but since race 2 is pathogenic on tomato, *VdAve1* is clearly not a determinant of pathogenicity on tomato. Many other genes of *V. dahliae* that contribute to virulence have been reported (de Sain and Rep 2015; Klimes et al. 2015). Nevertheless, we still do not know how pathogenicity and host range of *V. dahliae* are determined.

Identification of the genes involved in pathogenicity on specific host plants and elucidation of the host-determining mechanisms of *V. dahliae* is crucial for developing control methods for verticillium wilt in various plants. In this study, we isolated genetic recombinants between pathogenic and nonpathogenic strains of *V. dahliae* using protoplast fusion to search for the specific genomic region(s) and genetic sequence(s) determining pathogenicity on tomato.

Materials and methods

Protoplast preparation

Strains of *V. dahliae* were shake-cultured in potato sucrose broth (made from fresh potatoes) for 1 week at 25 °C in the dark. Fungal conidia were collected by centrifugation (1500 × *g*) after removing mycelia by filtering through a single layer of cheesecloth. Then 10^9 conidia were cultured in 1 L of PDY broth [potato dextrose broth including 0.5% (w/v) Bacto Yeast Extract; both from BD, Franklin Lakes, NJ, USA] with gentle stirring for 18 h at 25 °C in the dark. Mycelia were collected using nylon mesh (50 µm) and were resuspended in a solution of 0.5% (w/v) Driselase (Sigma-Aldrich, St. Louis, MO, USA) and 0.5% (w/v) of Lysing Enzymes (Sigma-Aldrich) in 1.2 M $MgSO_4$, then shaken gently (40 rpm) for 4 h. After centrifugation (1500 × *g*, 5 min), floating protoplasts were collected and resuspended in STC (1 M sorbitol, 10 mM Tris–HCl pH 7.5, 10 mM $CaCl_2$). The protoplast suspension was filtered through nylon mesh (25 µm) to remove hyphal fragments, and protoplasts were collected by centrifugation (1000 × *g*, 5 min).

Fungal transformation

V. dahliae strains TV103 (pathogenic on tomato) and Cns (nonpathogenic on tomato) (Usami and Amemiya 2005; Usami et al. 2007) were transformed, respectively, using plasmid pUCH1 (Bej and Perlin 1989) bearing the hygromycin B-resistance gene, and pII99 (Namiki et al. 2001) bearing the geneticin-resistance gene. Each linearized plasmid (10 µg) was mixed with the respective protoplast suspension (5×10^7 protoplasts in 100 µL of STC) and incubated for 20 min on ice, then 1 mL of polyethylene glycol (PEG) solution [20% (w/v) PEG6000 in STC] was gradually added to the protoplast suspension and mixed gently. Immediately, 3 mL of STC was mixed in gently, and protoplasts were recovered by centrifugation (1000 × *g*, 5 min). Protoplasts were resuspended in molten sPSA [potato sucrose agar (PSA) including 1 M sucrose; 48 °C] and dispensed into plastic plates. The plates were incubated at 25 °C for 12 h in the dark, then overlaid with an equal volume of PSA including 100 µg/mL of hygromycin B or geneticin. Plates were incubated at 25 °C for several days in the dark. Single-conidium isolates of generated transformants were cultured on PSA including 50 µg/mL of hygromycin B or geneticin.

Genetic recombination by protoplast fusion

Protoplasts of strain HR2 (hygromycin B-resistant transformant derived from strain TV103, pathogenic on tomato) and GR8 (geneticin-resistant transformant derived from strain Cns, nonpathogenic on tomato) were prepared. Protoplast suspensions (5×10^7 protoplasts in 100 µL of STC) of these two strains were mixed, 2 mL of PEG solution gradually added, and the suspension incubated for 30 min at 25 °C. After 10 mL of STC was mixed into the suspension, the protoplasts were recovered by centrifugation (1,000 × *g*, 5 min), then resuspended in molten sPSA (48 °C) and dispensed into plastic plates. The plates were incubated at 25 °C for 12 h in the dark, then overlaid with an equal volume of PSA including both hygromycin B (100 µg/mL) and geneticin (100 µg/mL). Plates were incubated at 25 °C for several days in the dark. A single conidium was isolated from each colony on the plate and cultured on PSA including both hygromycin B (50 µg/mL) and geneticin (50 µg/mL).

Pathogenicity test

Conidial suspensions (10^9 conidia in 100 mL of sterile distilled water) of parental and fusion strains of *V. dahliae* were prepared as described in the Protoplast preparation section. Five 2-week-old seedlings each of tomato (*Solanum lycopersicum* cv. Kyouryoku-beiju) and eggplant (*Solanum melongena* cv. Senryo-nigo) were inoculated with a strain by dipping the roots into a conidial suspension for 1 min. These inoculated seedlings, plus five uninoculated seedlings each of tomato and eggplant, were planted in commercial potting soil (Genkikun Kasai 200; Katakura and Co-op Agri Corporation, Tokyo, Japan) and maintained in a growth chamber at 25 °C with a 12 h photoperiod. One month after inoculation, the severity of foliar symptoms on each plant was scored on a scale of 0–3, where 0 = no symptoms, 1 = yellowed or wilted part affecting < 1/3 of leaf area, 2 = yellowed or wilted affecting 1/3 to 2/3 of leaf area, and 3 = yellowed or wilted

part affecting > 2/3 of leaf area. A foliar symptom index was then calculated for each plant as $[(\Sigma F_i \times L_i)/(3 \times L_t)] \times 100$, where F_i is the foliar severity score, L_i is number of leaves with F_i, and L_t is the total number of leaves scored for the plant. An average index for the five plants was then calculated. The severity of vascular symptoms in the hypocotyl was scored on a scale of 0–3, where 0 = no vascular discoloration, 1 = slight discoloration, 2 = moderate discoloration, and 3 = severe discoloration. A vascular severity index was then calculated as $[(\Sigma V_i \times P_i)/(3 \times P_t)] \times 100$, where V_i is the vascular severity score, P_i is the number of plants with V_i, and P_t is the total number of plants. The pathogen was then reisolated from the middle of each hypocotyl as described previously (Usami et al. 2017).

Southern blotting and PCR assays of fusion strains

Fungal genomic DNA was extracted as described by Usami et al. (2002). The chromosome constitution of fusion strains of *V. dahliae* was analyzed by genomic Southern hybridization probed with telomere sequences as described previously (Usami et al. 2017). The existence of sequences in the genome of each fusion strain was investigated by PCR assay using DNA markers specific to the genome of parental strain TV103, which is pathogenic on tomato. Previously reported microsatellite markers (Almany et al. 2009; Barbara et al. 2005; Berbegal et al. 2011; Li et al. 2013) and primer pair *VdAve1F*/*VdAve1R*, that amplifies effector gene *VdAve1* (primers, de Jonge et al. 2012), were used as DNA markers and amplified using the PCR programs as described in the cited references. In addition, we used the strain-specific PCR primers designed by Usami and Amemiya (2007) for amplified fragment length polymorphism (AFLP) analyses to compare genomic sequences of various *V. dahliae* strains, which differed in pathogenicity in some plant species. Consequently, they found several strain-specific genomic sequences. Cycling conditions for the PCR amplification with those primers were 95 °C for 5 min, (95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s) × 30 cycles, and 72 °C for 5 min. PCR assays were done at least three times to confirm the results.

Chromosomal mapping of TV103 and design of DNA markers

Draft genome sequencing of *V. dahliae* strain TV103, a strain pathogenic on tomato, was performed by Genome Sequencer-FLX (Roche, Basel, Switzerland). Preparation of mate-pair library (fragment length: 8 kb), analysis by Genome Sequencer-FXL, assembly by software GS De Novo Assembler ver. 2.8 (Roche), and mapping by software GS Reference Mapper ver. 2.8 (Roche) were done at Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan). Connection of the genomic sequence at the scaffold gap was verified by PCR amplification across the gap or by cloning the DNA fragment containing the gap from the genomic library of strain TV103 constructed in the fosmid vector pCC1FOS (Epicentre, Madison, WI, USA). DNA markers (PCR primers for selective amplification of genomic sequence of strain TV103) were designed based on the genomic map of TV103. The nucleotide sequence of strain TV103 was compared with the partial sequence of the corresponding genomic region of parental strain Cns, which is nonpathogenic on tomato. PCR primers were designed on the basis of a difference in sequence between the two strains. The PCR using these DNA markers was performed as described for the DNA markers in section Southern blotting and PCR assays of fusion strains.

Statistical analyses

Severity of foliar and vascular symptoms was compared using Mann–Whitney *U* tests. Frequency of pathogen reisolation from inoculated plants was compared using Fisher's exact test. Statistical analyses were performed using EZR v. 1.40 (Kanda 2013; Saitama Medical Center, Jichi Medical University; https://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html).

Results

Genetic recombination by protoplast fusion

Protoplast fusion between the two strains of *V. dahliae*, HR2 (hygromycin B-resistant, pathogenic on tomato) and GR8 (geneticin-resistant, nonpathogenic on tomato), resulted in 20 fusion strains (HGI1–20) capable of growing on PSA containing both hygromycin B and geneticin. When the chromosomal constitution of both the parental and fusion strains was analyzed by genomic Southern hybridization probed with telomere sequences at both ends of each chromosome, one pair of bands of unique size was detected per chromosome for each strain. The banding patterns of the two parental strains (Fig. 1, lanes H and G) differed greatly, indicating that the chromosomes of these strains appear to have a specific structure. The banding patterns of the fusion strains (Fig. 1, lanes 1–20) consisted of combinations of bands from the parental strains. The number of detected bands did not differ greatly among the parental strains and the fusion strains.

Pathogenicity tests of fusion strains

On inoculated tomato plants, eight of the 20 fusion strains caused foliar symptoms that were significantly more severe

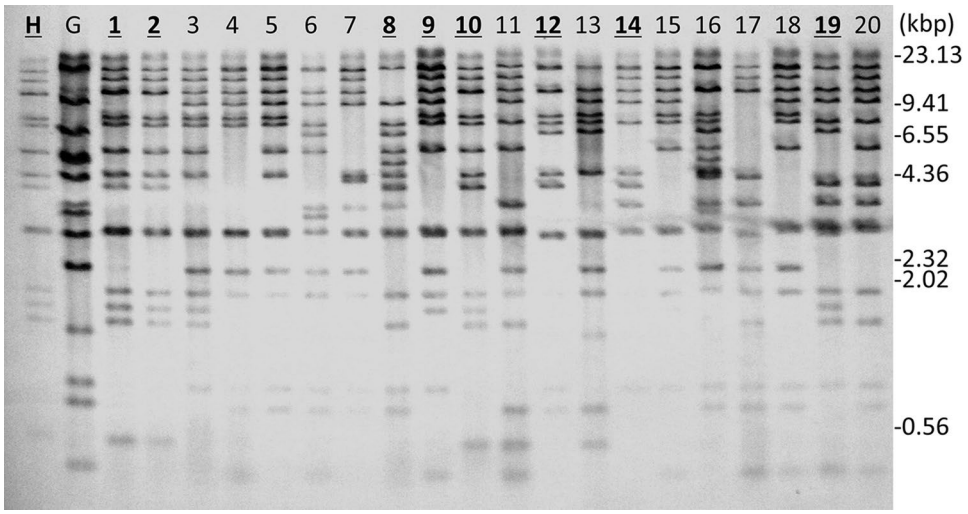

Fig. 1 Genomic Southern hybridization of parental and fusion strains of *Verticillium dahliae* probed with telomere sequences $(TTAGGG)_5$. Each genomic DNA was digested with restriction enzyme HindIII. *H* HR2 (hygromycin B-resistant transformant derived from strain TV103, pathogenic on tomato). *G* GR8 (geneticin-resistant transformant derived from strain Cns, nonpathogenic on tomato). Lane numbers indicate respective fusion strains HGI1–20. Strains pathogenic on tomato are indicated by bolded underlined text

than those produced by GR8 (the nonpathogenic parental strain) (Table 1), and growth of these tomato plants was suppressed. Vascular symptoms were often observed on tomato plants with no foliar symptoms or growth suppression. In addition, pathogens were frequently reisolated from hypocotyl tissues of symptomless tomato plants. The eight fusion strains that induced significant foliar symptoms on tomato (HGI1, 2, 8, 9, 10, 12, 14, and 19) were thus considered to be pathogenic on tomato. In contrast to the strains on tomato, all the fusion strains and both parental strains were highly virulent on eggplant (Table 2). Pathogenicity of each fusion strain was verified by repetitive inoculation tests.

Analysis of fusion strains using DNA markers

The parental origin of the genomic sequences in each fusion strain was investigated by PCR assay using parent-specific genomic DNA markers. First, the fusion strains were screened by PCR for DNA markers specific to the parent strain HR2 (pathogenic on tomato). Twenty-eight primers were then selected from previously reported DNA markers (see Materials and methods); their nucleotide sequences are presented in Table S1. In the subsequent PCR assay of the 20 fusion strains using the selected 28 DNA markers (see Fig. 2a–f for representative results of PCR assays). Two of the amplification patterns, from DNA markers T12 and VDA787, agreed with the pathogenicity of each strain on tomato (Fig. 2a, b). A PCR product (approximately 600 bp) was amplified in the pathogenic parental strain (HR2) by DNA marker T12, but not in the nonpathogenic parental strain (GR8). This PCR product was amplified only in the eight fusion strains that were pathogenic on tomato (Fig. 2a). Additionally, the primer pair for DNA marker VDA787 amplified a PCR product of approximately 160 bp in the pathogenic parental strain (HR2), whereas a product with fewer base pairs was amplified in the nonpathogenic parental

Table 1 Pathogenicity of parental and fusion strains of *Verticillium dahliae* on tomato

Inoculum	Symptom severity[a]		Frequency of reisolation[b]
	Foliar	Vascular	
Parental strain			
HR2	61.1*	100.0*	5/5
GR8	0.0	0.0	3/5
Fusion strain			
HGI1	69.7*	100.0*	5/5
HGI2	68.0*	100.0*	5/5
HGI3	3.0	6.7	4/5
HGI4	0.0	20.0	3/5
HGI5	4.3	40.0*	4/5
HGI6	1.1	26.7*	5/5
HGI7	0.0	13.3	4/5
HGI8	68.3*	80.0*	5/5
HGI9	78.7*	100.0*	5/5
HGI10	73.7*	80.0*	5/5
HGI11	3.8	33.3*	4/5
HGI12	61.7*	100.0*	5/5
HGI13	7.8	26.7*	4/5
HGI14	32.8*	93.3*	5/5
HGI15	2.7	40.0*	5/5
HGI16	0.0	20.0	3/5
HGI17	0.0	0.0	4/5
HGI18	0.0	13.3	4/5
HGI19	88.3*	100.0*	5/5
HGI20	1.1	33.3*	5/5
Uninoculated	0.0	0.0	0/5*

[a] Average index for foliar and vascular symptoms is from five plants. Asterisk denotes a significant difference compared with nonpathogenic parent strain GR8 in a Mann–Whitney *U* test ($P < 0.05$)

[b] Frequency of pathogen reisolation from hypocotyls (number of isolations from inoculated plants/total number of inoculated plants). Asterisks denote a significant difference compared with the nonpathogenic parent strain GR8 in Fisher's exact test ($P < 0.05$)

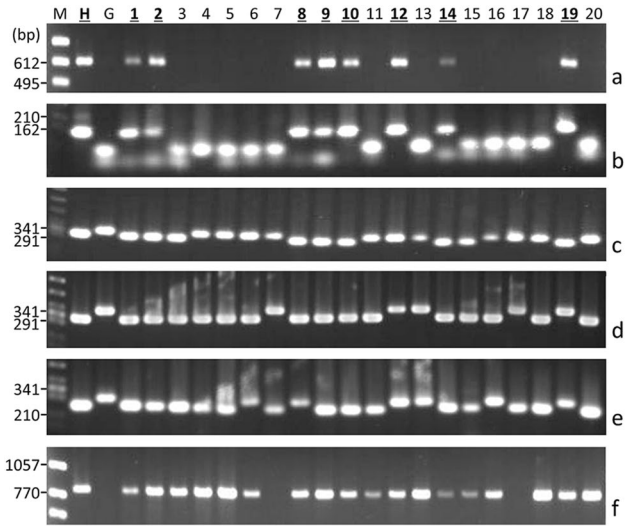

Fig. 2 PCR results using primers for DNA markers and genomic DNA of parental and fusion strains of *Verticillium dahliae*. **a–f**: DNA markers T12, VDA787, VD12, VD65, VD92, and *VdAve1*, respectively. *M* molecular size marker φX174 *Hinc*II. H: HR2 (hygromycin B-resistant transformant derived from strain TV103, pathogenic on tomato). *G* GR8 geneticin-resistant transformant derived from strain Cns, nonpathogenic on tomato). Lane numbers indicate respective fusion strains HGI1–20. Strains pathogenic on tomato are indicated by bolded, underlined text

Fig. 3 Map of chromosome 3 of *Verticillium dahliae* JR2 (de Jonge et al. 2013) and genomic sequence of *V. dahliae* TV103. Each white arrowhead indicates the position of a DNA marker. The white box in chromosome 3 of JR2 indicates the centromere-like AT-rich sequence. The gray bars indicate continuous genomic regions of TV103 verified by genomic sequencing, cloning, and PCR amplification

strain (GR8). The larger (160 bp) PCR product, similar to that for the pathogenic parent HR2, was amplified only in the eight fusion strains that were pathogenic on tomato (Fig. 2b).

Mapping of DNA markers

The genomic sequence of *V. dahliae* strain TV103 (pathogenic on tomato) was analyzed and scaffolds containing nucleotide sequences of the two DNA markers T12 and VDA787 were identified and mapped to the whole-genome data of *V. dahliae* reference strain JR2 (de Jonge et al. 2013). Physical continuity of these scaffolds with other scaffolds on the map was confirmed by PCR assay or genomic cloning of border sequences of each scaffold (Fig. 3). DNA markers T12 and VDA787 were respectively mapped on the genomic regions corresponding to the long arm of chromosome 3 of *V. dahliae* JR2.

Analysis using additional DNA markers and fusion strains

Additional DNA markers specific to the parent strain TV103, which was used for genetic recombination of *V. dahliae*, were designed based on the genomic location corresponding to chromosome 3 of strain JR2. The position and primer sequence of these DNA markers (Ch3-TV7, Ch3-TV9, Ch3-TV11, and Ch3-TV12) are presented in Fig. 3 and Table S1, respectively. In the PCR analysis of the 20 fusion strains (HGI1–20) obtained from protoplast fusion between HR2 and GR8 (Fig. 4) using these markers, DNA marker Ch3-TV7 was present even in fusion strains HGI3 and 15 that were nonpathogenic on tomato. On the other hand, markers Ch3-TV7 and Ch3-TV12 were not amplified, even in fusion strains HGI12 and HGI19 that were pathogenic on tomato. However, amplification of Ch3-TV11 and Ch3-TV9 completely accorded with pathogenicity of strains on tomato (as had DNA markers VDA787 and T12). Fifteen additional fusion strains were generated in another fusion trial between HR2 and GR8, their pathogenicity tested on tomato and eggplant (Table S2), and their DNA markers analyzed (Fig. 5). When the pathogenicity on tomato and the DNA marker results for all 35 fusion strains (20 original and 15 additional) were collated (Table 3), only one DNA marker Ch3-TV11 uniquely agreed with the pathogenicity results of the fusion strains on tomato.

Discussion

Here, genetic recombination between a tomato-pathogenic and a nonpathogenic strain of *V. dahliae* was achieved through protoplast fusion. Genetic recombination in asexual *V. dahliae* via a parasexual cycle has been previously reported (O'Garro and Clarkson 1988, 1992; Typas and Heale 1978; Usami and Amemiya 2005), but our report is the first on using genetic recombination to identify pathogenicity-determining chromosomal region(s). In the first experiment in this study, 20 fusion strains (HGI1–20) were generated, and banding patterns of the fusion strains, probed with telomere sequences in genomic Southern hybridization tests, indicated that these strains had combinations of chromosomes from the two parental strains (Fig. 1), and the number of bands in each fusion strain was similar to that in the parental strains. Thus, each fusion strain was haploid. An

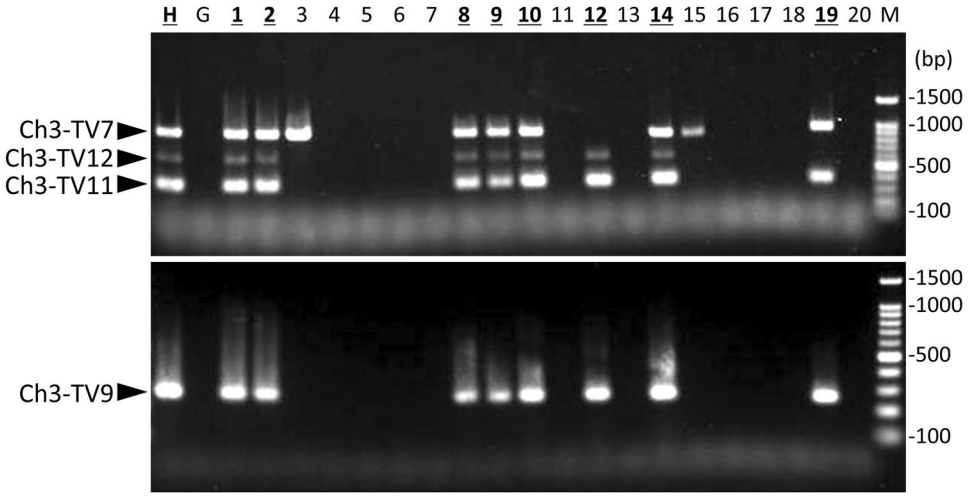

Fig. 4 PCR results using primers of each DNA marker (Ch3-TV7, Ch3-TV9, Ch3-TV11, and Ch3-TV12) and genomic DNA of parental and fusion strains of *Verticillium dahliae*. *M* 100 bp ladder marker. H: HR2 (hygromycin B-resistant transformant derived from strain TV103, pathogenic on tomato). *G* GR8 (geneticin-resistant transformant derived from strain Cns, nonpathogenic on tomato). Lane numbers indicate respective fusion strains. Strains pathogenic on tomato are indicated by bolded, underlined text

Table 2 Pathogenicity of parental and fusion strains of *Verticillium dahliae* on eggplant

Inoculum	Symptom severity[a]		Frequency of reisolation[b]
	Foliar	Vascular	
Parental strain			
HR2	90.6	100.0	5/5
GR8	88.3	93.3	5/5
Fusion strain			
HGI1	85.8	100.0	5/5
HGI2	94.7	93.3	5/5
HGI3	97.8	100.0	5/5
HGI4	98.3	100.0	5/5
HGI5	87.8	100.0	5/5
HGI6	81.3	86.7	5/5
HGI7	93.3	93.3	5/5
HGI8	83.4	100.0	5/5
HGI9	95.0	93.3	5/5
HGI10	96.1	100.0	5/5
HGI11	87.3	100.0	5/5
HGI12	91.7	93.3	5/5
HGI13	90.4	100.0	5/5
HGI14	83.0	100.0	5/5
HGI15	86.3	100.0	5/5
HGI16	80.3	93.3	5/5
HGI17	81.1	93.3	5/5
HGI18	87.8	100.0	5/5
HGI19	91.7	100.0	5/5
HGI20	86.3	100.0	5/5
Uninoculated	0.0*	0.0*	0/5*

[a] Average index for foliar and vascular symptoms is from five plants. Asterisk denotes a significant difference compared with nonpathogenic parent strain GR8 in a Mann–Whitney *U* test ($P < 0.05$)

[b] Frequency of pathogen reisolation from hypocotyls (number of isolations from inoculated plants/total number of inoculated plants). Asterisks denote a significant difference compared with the nonpathogenic parent strain GR8 in Fisher's exact test ($P < 0.05$)

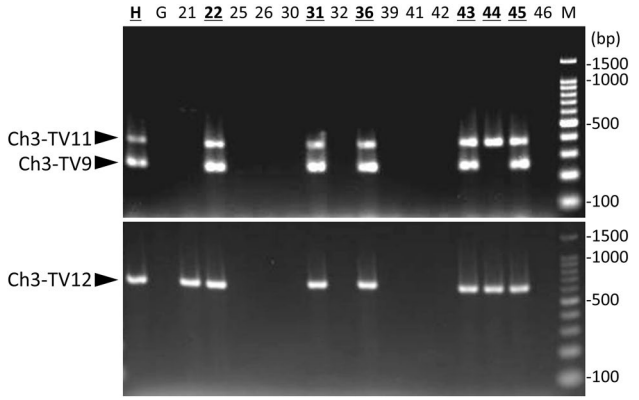

Fig. 5 PCR results using primers for each DNA marker (Ch3-TV9, Ch3-TV11, and Ch3-TV12) and genomic DNA of parental and fusion strains of *Verticillium dahliae*. *M* 100 bp ladder marker. H: HR2 (hygromycin B-resistant transformant derived from strain TV103, pathogenic on tomato). *G* GR8 (geneticin-resistant transformant derived from strain Cns, nonpathogenic on tomato). Lane numbers indicate respective fusion strain. Strains pathogenic on tomato are indicated by bolded, underlined text

additional 15 fusion strains were generated to produce a total of 35 strains. Only 14 of the 35 strains were pathogenic on tomato, whereas all 35 were pathogenic on eggplant. These results indicated that a portion of the genomic region from parental strain HR2 (pathogenic on tomato) had been inherited by these 14 fusion strains; this region likely includes the gene(s) involved in the pathogenicity of *V. dahliae* on tomato.

In the first 20 fusion strains that were generated, PCR results for DNA markers T12 and VDA787 completely agreed with the pathogenicity of the strains on tomato (Fig. 2). These notable DNA markers were mapped on chromosome 3 of *V. dahliae* JR2, a strain whose genomic sequence and map have been published (de Jonge et al. 2013). The genomic sequence composition of each

Table 3 Pathogenicity of parental and fusion strains of *Verticillium dahliae* on tomato and genotype of parental and fusion strains based on DNA markers

		DNA marker[b]									
Strain	Pathogenicity on tomato[a]	VDA 787	T12	Ch3-TV9	Ch3-TV11	Ch3-TV12	Ch3-TV7	VD 12	VD 65	VD 92	*Vd Ave1*
Parental											
HR2	+	A	A	A	A	A	A	A	A	A	A
GR8	-	B	B	B	B	B	B	B	B	B	B
Fusant											
HGI1	+	A	A	A	A	A	A	A	A	A	A
HGI2	+	A	A	A	A	A	A	A	A	A	A
HGI8	+	A	A	A	A	A	A	A	A	B	A
HGI9	+	A	A	A	A	A	A	A	A	A	A
HGI10	+	A	A	A	A	A	A	A	A	A	A
HGI14	+	A	A	A	A	A	A	A	A	A	A
HGI31	+	A	A	A	A	A	A	B			
HGI36	+	A	A	A	A	A	A	B			
HGI43	+	A	A	A	A	A	A	B			
HGI12	+	A	A	A	A	A	B	B	B	B	A
HGI22	+	A	A	A	A	A	B	B			
HGI45	+	A	A	A	A	A	B	B			
HGI19	+	A	A	A	A	B	A	A	B	B	A
HGI44	+	B	B	B	A	A	A	A			
HGI21	-	B	B	B	B	A	A	A			
HGI3	-	B	B	B	B	B	A	A	A	A	A
HGI15	-	B	B	B	B	B	A	A	A	A	A
HGI26	-	B	B	B	B	B	A	A			
HGI41	-	B	B	B	B	B	A	A			
HGI4	-	B	B	B	B	B	B	B	A	A	A
HGI5	-	B	B	B	B	B	B	B	A	A	A
HGI6	-	B	B	B	B	B	B	B	A	B	A
HGI7	-	B	B	B	B	B	B	B	B	A	B
HGI11	-	B	B	B	B	B	B	B	A	A	A
HGI13	-	B	B	B	B	B	B	B	B	B	A
HGI16	-	B	B	B	B	B	B	B	A	B	A
HGI17	-	B	B	B	B	B	B	B	B	A	B
HGI18	-	B	B	B	B	B	B	B	A	A	A
HGI20	-	B	B	B	B	B	B	B	A	A	A
HGI25	-	B	B	B	B	B	B	B			
HGI30	-	B	B	B	B	B	B	B			
HGI32	-	B	B	B	B	B	B	B			
HGI39	-	B	B	B	B	B	B	B			
HGI42	-	B	B	B	B	B	B	B			
HGI46	-	B	B	B	B	B	B	B			

[a] Presence (+) or absence (-) of a significant difference in foliar symptoms on tomato by Mann–Whitney *U*-test ($P < 0.05$) compared to nonpathogenic parent strain GR8. Detailed data for pathogenicity tests are in Tables 1 and S1.

[b] A: The strain has the same genomic sequence as strain HR2, pathogenic on tomato. B: The strain has the same genomic sequence as strain GR8, nonpathogenic on tomato. If a cell in the table has no entry, pathogenicity was not tested.

chromosome often differs among strains of *V. dahliae* (de Jonge et al. 2013; Usami et al. 2008). Therefore, we verified that the structure of a 2-Mbp genomic region including DNA markers T12 and VDA787 in *V. dahliae* TV103 was similar to that in JR2 through draft genome sequencing and mapping (Fig. 3). In the PCR analyses, five markers in that genomic region (VDA787, T12, Ch3-TV9, Ch3-TV11, and Ch3-TV12) tended to be specific to fusion strains that were pathogenic on tomato (Table 3), supporting the supposition that the 2-Mbp region was continuous in the genomic DNA in strain TV103. However, these five markers were not linked in some fusion strains. For example, strain HGI44 had two of the five DNA marker sequences (Ch3-TV11 and Ch3-TV12) but lacked the other three. This strain has the same sequence of the codominant DNA marker VDA787 as in the nonpathogenic parent strain GR8; therefore, the loss of the DNA marker sequences in HGI44 might occur by chromosomal recombination between markers rather than by deletion. Chromosomal recombination via a parasexual cycle has been reported not only in *V. dahliae* (Typas and Heale 1978), but also in *Fusarium oxysporum* (Teunissen et al. 2002, 2003) and appears to be ubiquitous in asexual fungi. We exploited the fusion strains with recombined chromosomes to identify genomic region(s) that were in complete accord with pathogenicity and found only DNA marker Ch3-TV11 that completely matched the pathogenicity on tomato (Table 3). Therefore, we inferred that the genomic sequence involved in pathogenicity was near marker Ch3-TV11 (a ~ 1-Mbp region between DNA marker Ch3-TV9 and Ch3-TV12).

Some fusion strains (such as HGI5, HGI11, HGI13, and HGI15) lacking the genomic region including DNA marker Ch3-TV11 (Ch3-TV11 region) induced slight foliar symptoms and significant vascular symptoms in inoculated tomato plants. Thus, genomic regions other than the Ch3-TV11 region may be partially involved in the virulence in tomato. On the other hand, fusion strains HGI14 and HGI31, bearing the Ch3-TV11 region, appeared less virulent on tomato but highly virulent on eggplant. O'Garro and Clarkson (1992) reported that recombinants that were obtained from genetic recombination between two *V. dahliae* strains pathogenic on tomato were less virulent than the parental strains. They suggested that virulence of *V. dahliae* on tomato may be controlled polygenically, and the lower virulence of fusion strains HGI14 and HGI31 seen in this study supports this suggestion. Effector gene *VdAve1* (de Jonge et al. 2012) is a known virulence factor for *V. dahliae* on tomato. However, strains HGI14 (Fig. 2) and HGI31 (data not shown) carry *VdAve1* in their genome; therefore, the loss of *VdAve1* cannot be the cause of the reduced virulence of these strains. PCR amplification of *VdAve1* did not agree with the pathogenicity of the fusion strains (Fig. 2); in fact, many strains carrying *VdAve1* were nonpathogenic on tomato. However, all the highly virulent strains carried *VdAve1* without exception, indicating the possibility that *VdAve1* does play a role in their virulence on tomato.

Klosterman et al. (2011) and de Jonge et al. (2013) inferred that effector genes in a lineage-specific genomic region (LS region) contribute to virulence of *V. dahliae* in plants. Chen et al. (2018) also reported that genes in the LS region are involved in specific virulence on cotton. There are many LS regions (up to 4 Mbp in total) in the genome of *V. dahliae* strains (de Jonge et al. 2013; Klosterman et al. 2011). However, there are not many lineage-specific sequences in the Ch3-TV11 region.

Future studies could narrow the candidates for the genomic region responsible for the pathogenicity of *V. dahliae* on tomato using more fusion strains and DNA markers. Moreover, pathogenicity-determining gene(s) might be identified through functional analyses such as targeted disruption of candidate sequences in that genomic region. In our previous genetic analysis to investigate the genomic region responsible for the pathogenicity of *V. dahliae* on sweet pepper (*Capsicum annuum*) using the same method used in the current study, we found a DNA marker in accord with pathogenicity of genetic recombinants (Imano et al. 2018). Investigating the genomic sequences involved in the pathogenicity on sweet pepper as well as on tomato will help elucidate the system that determines the host range of *V. dahliae*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights statement This article does not contain any studies with human participants or animals performed by any of the authors.

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