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FUNGAL DISEASES

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Cloning of DNA fragments specific to the pathotype and race of *Verticillium dahliae*

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Abstract Japanese isolates of *Verticillium dahliae*, a causal agent of wilt disease in many plants, are classifiable into pathotypes based on their pathogenicity. Because these pathotypes are morphologically indistinguishable, establishing a rapid identification method is very important for the control of this pathogen in Japan. For cloning DNA fragments that are useful for identification and specific detection of *V. dahliae* pathotypes, we performed random amplified polymorphic DNA (RAPD) analyses using various isolates. One polymerase chain reaction (PCR) product, E10-U48, was specific to isolates pathogenic to sweet pepper. The other product, B68-TV, was specific to race 1 of isolates pathogenic to tomato. The specificity of these sequences was confirmed by genomic Southern hybridization. Further analyses revealed that the region peripheral to B68-TV obtained from the genomic DNA library includes the sequence specific to all isolates pathogenic to tomato (races 1 and 2). Moreover, sequence tagged site (STS) primers designed from B68-TV and its peripheral region showed race-specific and pathotype-specific amplification in a PCR assay. The probes and primers obtained in this study are likely to be useful tools for the identification and specific detection of pathotypes and races of *V. dahliae*.

Key words *Verticillium dahliae* · Pathotype · Race · RAPD · DNA marker · PCR

Introduction

Verticillium dahliae Klebahn is a soilborne fungal pathogen that causes wilt disease in plants (Pegg and Brady 2002). Although this fungus is polyxenic, the host range of each isolate typically differs. Hagiwara (1990) proposed dividing isolates of *V. dahliae* into tomato, sweet pepper, and eggplant pathotypes based on their pathogenicity to these representative host species of solanaceous plants. All these pathotypes are uniformly pathogenic to eggplant. On the other hand, tomato and sweet pepper are specifically invaded by tomato and sweet pepper pathotypes, respectively. However, three isolates that are pathogenic to both tomato and sweet pepper have been reported (Iijima 1983; Oshima et al. 1993; Shiraishi and Nieda 1995). These isolates have been classified as a new group, tomato–sweet pepper pathotype (Hagiwara 1990). Isolates of *V. dahliae* that are pathogenic to tomato are divided into two races. Although race 1 isolates are not highly virulent on tomato cultivars carrying resistance gene *Ve*, race 2 isolates can overcome this resistance gene (Brammall 1989).

In recent years, the polymerase chain reaction (PCR) technique has been used to identify fungal species and pathotypes. It is a method that can replace morphological observation and time-consuming inoculation tests. Some species-specific PCR primers of *V. dahliae* have been designed from ribosomal DNA (Nazar et al. 1991; Li et al. 1994) or other genomic sequences (Li et al. 1999). In addition, Pérez-Artés et al. (2000) developed PCR primers that can differentiate defoliating and nondefoliating pathotypes of cotton.

Usami et al. (2001) identified a pathotype-specific gene, *vdt1*, in the genomic DNA fragment specific to tomato pathotype of *V. dahliae* cloned by Amemiya et al. (2000). They designed a pair of PCR primers to identify and specifically detect the tomato pathotype using sequence *vdt1* (Usami et al. 2002). However, an isolate of tomato–sweet pepper pathotype, Vdp4, was not detected by these PCR primers because it does not have the *vdt1* sequence (unpublished data). Therefore, a new DNA sequence was needed for distinguishing isolates pathogenic to tomato with certainty. This study compares the composition of genomic DNA among pathotypes of *V. dahliae* using random amplified polymorphic DNA (RAPD) analysis to obtain DNA fragments that are specific to each pathotype.

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The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under accession number AB095266.

Materials and methods

Fungal isolates and DNA extraction

Fungal isolates used in this study are listed in Table 1. All fungal isolates were maintained on potato sucrose agar slants. For preparation of budding spores, each isolate was cultured in potato semisynthetic broth (potato broth with 0.005% calcium nitrate, 0.2% disodium hydrogenphosphate, 0.5% peptone, 2% sucrose) with shaking at 25°C for 1 week. The culture was filtered through gauze to remove hyphae; spores were collected by centrifugation (3000*g* for 5 min). Spores were suspended in 50% glycerol and stored at –40°C until use. Fungal genomic DNAs were extracted following the method of Usami et al. (2002).

RAPD analysis

Forty-eight primers in DNA oligomer (12) set B-4, C-5, D-2, and E-1 (Wako, Osaka, Japan) were used for RAPD analysis of each isolate of *V. dahliae*. PCR amplification was performed in 50 µl of reaction mixture containing 100 ng fungal genomic DNA, 1 unit Ex *Taq* polymerase (Takara, Otsu, Japan), 1× Ex *Taq* reaction buffer (Takara), 0.25 mM each deoxyribonucleoside triphosphate and 100 pmol single primer using a PCR Thermal Cycler MP (Takara) programmed for 94°C for 3 min; 40 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min; and 72°C for 5 min. PCR products were electrophoresed on a 0.7% or 2.0% agarose gel in 1× TAE [40 mM Tris-acetate pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA)], stained with ethidium bromide and observed under ultraviolet (UV) irradiation.

Table 1. Isolates of *Verticillium dahliae* used in this study

Isolate	Isolated host	Location	Year	Pathotype[a]	Race	Pathogenicity	
						Tomato	Sweet pepper
TV103	Tomato	Tokyo	1971	Tomato	1	+	–
U22	*Aralia cordata*	Gunma	1986	Tomato	1	+	–
Shio	Tomato	Tokyo	Unknown	Tomato	1	+	–
TO2	Tomato	Gunma	1984	Tomato	1	+	–
TK23	Tomato	Kanagawa	1992	Tomato	1	+	–
Kgm	Tomato	Unknown	Unknown	Tomato	1	+	–
Gto2	Tomato	Gunma	2001	Tomato	1	+	–
84007	Okra	Unknown	Unknown	Tomato	1	+	–
G-39	Cabbage	Gunma	1994	Tomato	1	+	–
GM1	Melon	Gunma	Unknown	Tomato	1	+	–
TR-1	Tomato	Chiba	1985	Tomato	1	+	–
MIH004	Lettuce	Hyogo	2002	Tomato	1	+	–
Gto1	Tomato	Gunma	2001	Tomato	2	+	–
TO20	Tomato	Gunma	1991	Tomato	2	+	–
TO21	Tomato	Gunma	1991	Tomato	2	+	–
TO22	Tomato	Gunma	1991	Tomato	2	+	–
TO23	Tomato	Gunma	1991	Tomato	2	+	–
TO24	Tomato	Gunma	1991	Tomato	2	+	–
TO26	Tomato	Gunma	1991	Tomato	2	+	–
TK15	Tomato	Kanagawa	1992	Tomato	2	+	–
Gok1	Okra	Gunma	2001	Tomato	2	+	–
Yam1	Tomato	Yamanashi	Unknown	Tomato	2	+	–
Hok1	Tomato	Hokkaido	2003	Tomato	2	+	–
Gca1	Cabbage	Gunma	2001	Tomato	2	+	–
Vng	Tomato	Nagano	Unknown	Tomato	2	+	–
Vdp4	Sweet pepper	Nagano	1991	Tomato–sweet pepper	1[b]	+	+
U48	*Aralia cordata*	Gunma	1987	Sweet pepper	–	–	+
Cns	Eggplant	Nagano	1980	Sweet pepper	–	–	+
22210	Eggplant	Tokushima	1972	Sweet pepper	–	–	+
Vdp3	Sweet pepper	Nagano	1991	Sweet pepper	–	–	+
U20	*Aralia cordata*	Gunma	1986	Sweet pepper	–	–	+
P2-1	Sweet pepper	Hokkaido	1992	Sweet pepper	–	–	+
P2-2	Sweet pepper	Hokkaido	1992	Sweet pepper	–	–	+
P8-1	Sweet pepper	Hokkaido	1998	Sweet pepper	–	–	+
P9-1	Sweet pepper	Hokkaido	1999	Sweet pepper	–	–	+
P9-2	Sweet pepper	Iwate	1999	Sweet pepper	–	–	+
Ara406	*Aralia cordata*	Gunma	1977	Eggplant	–	–	–
Chr208	Chrysanthemum	Tokyo	1970	Eggplant	–	–	–
Ibh	Chinese cabbage	Ibaraki	Unknown	Eggplant	–	–	–
Y3-1	Eggplant	Yamagata	1972	Eggplant	–	–	–

[a] Pathotypes proposed by Hagiwara (1990)
[b] Revealed in this study

Cloning of RAPD products

Amplified RAPD products were purified using a Rapid PCR purification system (Marligen Biosciences, Ijamsville, MD, USA) inserted in plasmid vector pCR2.1-TOPO using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and used to transform competent cells of *Escherichia coli* strain JM109 (Takara). Ampicillin-resistant bacterial colonies on the plate were picked randomly and cultured; plasmid DNAs were extracted from bacterial cells. Each manipulation was performed following the method in the manufacturer's manuals or standard protocols. Extracted plasmids were cut with *Eco*RI and electrophoresed on a 1.0% TAE agarose gel; targeted PCR products were identified. The inserted DNA fragment of each plasmid was amplified by M13 forward/reverse PCR primers, and then used as a hybridization probe.

Construction of genomic DNA library and screening

Genomic DNA of *V. dahliae* TV103 was partially digested with *Sau*3AI, partially filled in the sticky end, ligated to Lambda BlueSTAR *Xho* I half-site arms (Novagen, San Diego, CA, USA), and packaged into the phage particles using Packagene lambda DNA packaging system (Promega, Madison, WI, USA). Phages were used to infect *E. coli* strain ER1647 and screening were performed by plaque hybridization. Positive plaques were recovered, and phages were used to infect *E. coli* strain BM25.8 for subcloning. This host strain converts the lambda DNA to a plasmid clone by a *Cre*/*loxP*-mediated recombination system. Plasmids were extracted from bacterial strain BM25.8 and used to transform host strain JM109, which is more suitable for large-scale preparation. Each manipulation was performed following the manufacturer's methods or standard protocols. Cloned genomic DNA fragment was digested by *Eco*RI and subcloned into pUC18. DNA inserts of each plasmid were amplified by M13 forward/reverse PCR primers and used as hybridization probes.

Southern blot hybridization

PCR products or fungal genomic DNAs digested with restriction enzyme were electrophoresed on 0.7% or 2.0% TAE agarose gel and transferred to a positively charged nylon membrane (Roche, Basel, Switzerland) using the method described by Selden (1990). A DIG DNA labeling and detection kit (Roche) was used for probe labeling with digoxigenin, hybridization, and detection of signals. Hybridization was performed in 5× SSC (SSC: 0.15 M NaCl, 15 mM sodium citrate), 0.1% sodium *N*-lauroyl sarcosinate, 0.02% sodium dodecyl sulfate (SDS), 20 µg/ml sheared DNA, and 1% blocking reagent (Roche) at 68°C. After the membranes were washed with 2× SSC, 0.1% SDS (at room temperature), 0.5× SSC, and 0.1% SDS (at 68°C), hybridization signals were detected.

Pathogenicity test

Three-week-old seedlings of tomato (*Lycopersicon esculentum* Mill.) cv. Momotaro (Ve^+) and cv. Oogata-fukuju (Ve^-) were inoculated by dipping roots into a spore suspension (10^9 spores in 100 ml water) of *V. dahliae* isolates. Inoculated plants were grown for 1 month in a greenhouse. Then the wilting score (0, none; 1, slight; 2, moderate; 3, severe) of each leaf was evaluated, and a disease index of each plant was calculated by the following formula: [total wilting score of a plant/(3 × total leaf number of a plant)] × 100. Pathogenicity of each fungal isolate was assessed using the average of the disease indices of nine plants used in the inoculation test.

PCR using sequence tagged site (STS) primer

The nucleotide sequence of a DNA fragment obtained from the genomic library was determined using a general autosequencer with a cycle sequencing protocol. Pairs of STS primers specific to pathotype and race were designed from this sequence. PCR amplification was performed in 50 µl of reaction mixture containing 100 ng fungal genomic DNA, 1 unit Ex *Taq* polymerase (Takara), 1× Ex *Taq* reaction buffer (Takara), 0.25 mM of each deoxyribonucleoside triphosphate and 100 pmol of each primer using a PCR Thermal Cycler MP (Takara) programmed for 94°C for 3 min; 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min; and 72°C for 5 min. PCR products were electrophoresed on a 0.7% TAE agarose gel, stained with ethidium bromide and observed under UV irradiation.

Results

RAPD analysis

We first performed RAPD analyses using only four isolates of each pathotype (tomato pathotype TV103, tomato–sweet pepper pathotype Vdp4, sweet pepper pathotype U48, and eggplant pathotype Chr208) to exclude ineffective RAPD primers. Among all four isolates, 35 of 48 primers yielded identical amplification patterns (Fig. 1A, B). Ten primers yielded specific amplification in tomato pathotype TV103 (Fig. 1C–E). Primer B63 yielded patterns specific to sweet pepper pathotype isolate U48 (F). These patterns and fungal pathogenicity to tomato and sweet pepper did not correspond. In contrast, primers E10 and B68 amplified the products specific to isolates pathogenic to sweet pepper (Fig. 1G, approximately 0.8 kbp) and tomato (Fig. 1H, approximately 1.3 kbp), respectively. In an expanded RAPD analysis using 25 isolates, primer E10 specifically amplified a PCR product (approximately 0.8 kbp) in isolates pathogenic to sweet pepper (Fig. 2). On the other hand, a PCR product (approximately 1.3 kbp) amplified with primer B68 was observed in tomato pathotype race 1 and tomato–sweet pepper pathotype (Fig. 3). A product of approximately 0.8 kbp, which was amplified from U48 by E10, was named

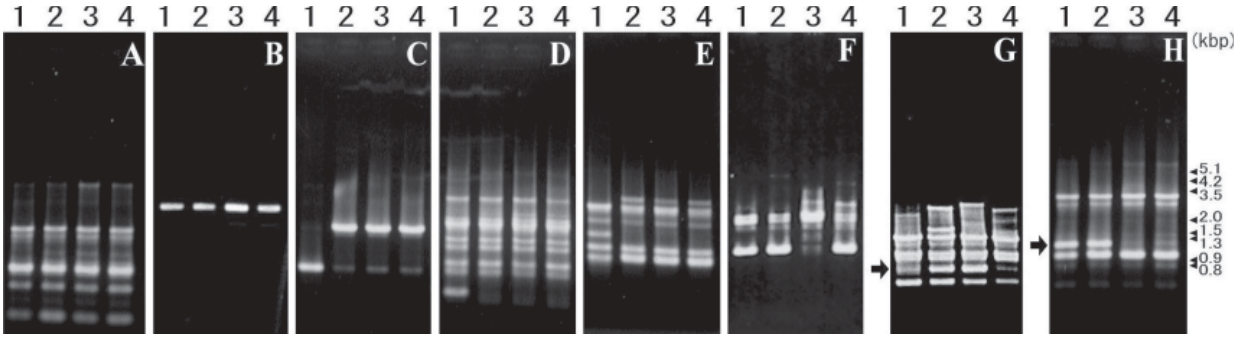

Fig. 1A–H. Agarose gel electrophoresis of random amplified polymorphic DNA (RAPD) products of *Verticillium dahliae*. Typical results among all analyses using 48 primers are shown. Lane *1*, tomato pathotype TV103; lane *2*, tomato–sweet pepper pathotype Vdp4; lane *3*, sweet pepper pathotype U48; lane *4*, eggplant pathotype Chr208. **A** Primer C85 and **B** primer E07 show no polymorphism; **C–E** primers B71, C84, and C88 are specific to tomato pathotype; **F** primer B63 specific to sweet pepper pathotype; **G** primer E10 specific to isolates that are pathogenic to sweet pepper; **H** primer B68 specific to isolates that are pathogenic to tomato. *Arrows* indicate the position of polymerase chain reaction (PCR) products E10-U48 (**G**, lane *3*) and B68-TV (**H**, lane *1*)

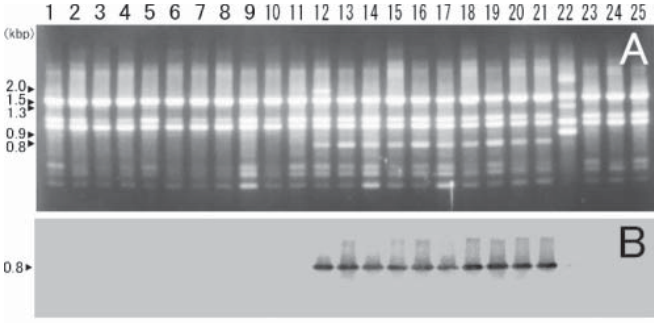

Fig. 2A, B. PCR products of RAPD analyses using primer E10 were electrophoresed on 2.0% agarose gel (**A**), and blotted and hybridized with E10-U48 (**B**). Lanes *1–5*, tomato pathotype race 1 (TV103, U22, Shio, TO2, TK23); lanes *6–11*, tomato pathotype race 2 (TO20, TO21, TO23, TO24, TO26, TK15); lane *12*, tomato–sweet pepper pathotype Vdp4; lanes *13–21*, sweet pepper pathotype (U48, Cns, Vdp3, 22210, U20, P2-1, P8-1, P9-1, P9-2); lanes *22–25*, eggplant pathotype (Y3-1, Chr208, Ibh, Ara406)

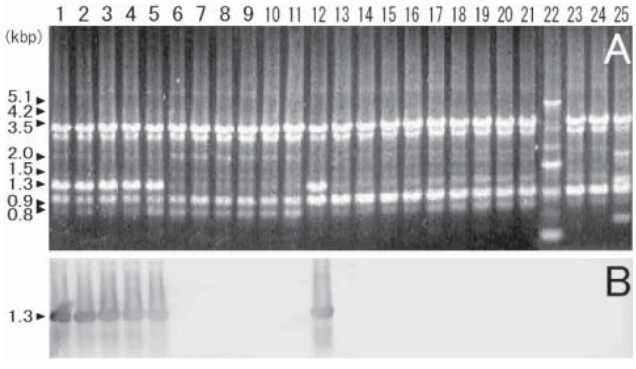

Fig. 3A, B. PCR products of RAPD analyses using primer B68 were electrophoresed on 0.7% agarose gel (**A**), and blotted and hybridized with B68-TV (**B**). Lanes *1–5*, tomato pathotype race 1 (TV103, U22, Shio, TO2, TK23); lanes *6–11*, tomato pathotype race 2 (TO20, TO21, TO23, TO24, TO26, TK15); lane *12*, tomato–sweet pepper pathotype Vdp4; lanes *13–21*, sweet pepper pathotype (U48, Cns, Vdp3, 22210, U20, P2-1, P8-1, P9-1, P9-2); lanes *22–25*, eggplant pathotype (Y3-1, Chr208, Ibh, Ara406)

E10-U48. Another of approximately 1.3 kbp, which was amplified from TV103 by B68, was named B68-TV. E10-U48 and B68-TV were cloned into the TA vector pCR2.1-TOPO.

Analysis of RAPD products

Genomic DNAs of each isolate were digested with *Bam*HI, electrophoresed, blotted, and hybridized with digoxigenin-labeled E10-U48 (Fig. 4) and B68-TV (Fig. 5). A clear band was detected specifically in isolates pathogenic to sweet pepper with the E10-U48 probe. The band sizes differed between tomato–sweet pepper and sweet pepper pathotype isolates. A conspicuous band specific to tomato pathotype race 1 and tomato–sweet pepper pathotype (approximately 3.5 kbp) and a faint band common to all isolates (between 4.3 and 6.5 kbp) were detected by B68-TV.

For further investigation, a DNA fragment of approximately 13.8 kbp (TomR1, DDBJ/EMBL/GenBank accession no. AB095266) was obtained from the genomic library of TV103 by plaque hybridization probed with B68-TV. TomR1 was digested into some restriction fragments and hybridized to *Bam*HI-digested fungal genomic DNA (Fig. 6). The multiple bands detected by probe A meant that many similar sequences exist even in the genomic DNA of eggplant pathotype. According to the restriction map for TomR1, two bands were expected. However, a band of approximately 3.5 kbp specific to tomato pathotype race 1 and tomato–sweet pepper pathotype was also detected by probe A (Fig. 6A). This appears to be the same as a band detected in the genomic Southern hybridization probed with B68-TV (Fig. 5). The nonspecific band detected by B68-TV (Fig. 5, between 4.3 and 6.5 kbp) was not observed in Fig. 6A. On the other hand, bands specific to all isolates that are pathogenic to tomato (tomato pathotype race 1, race 2, and tomato–sweet pepper pathotype) were detected by probes B, C, and D (Fig. 6B–D). In particular, a band larger than 9.4 kbp detected by probes C and D was conspicuous, while the band detected by probe B was very faint. The restriction

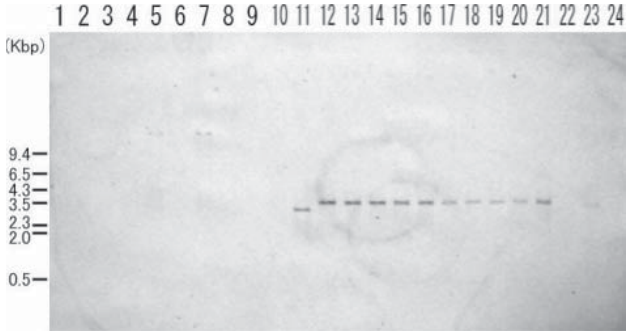

Fig. 4. Genomic Southern hybridization of *Verticillium dahliae* probed with E10-U48. Fungal genomic DNAs were digested with *Bam*HI. Lanes *1–6*, tomato pathotype race 1 (TV103, U22, Shio, TO2, TK23, Kgm); lanes *7–10*, tomato pathotype race 2 (Gto1, TO20, TO26, TK15); lane *11*, tomato–sweet pepper pathotype Vdp4; lanes *12–21*, sweet pepper pathotype (U48, Cns, 22210, Vdp3, U20, P2-1, P2-2, P8-1, P9-1, P9-2); lanes *22–24*, eggplant pathotype (Ibh, Chr208, Y3-1)

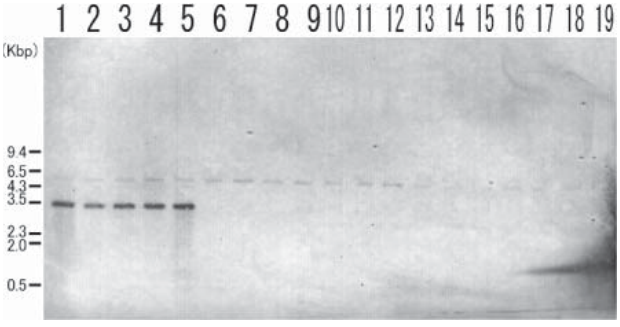

Fig. 5. Genomic Southern hybridization of *Verticillium dahliae* probed with B68-TV. Fungal genomic DNAs were digested with *Bam*HI. Lanes *1–4*, tomato pathotype race 1 (TV103, U22, Shio, TO2); lane *5*, tomato–sweet pepper pathotype Vdp4; lanes *6–11*, tomato pathotype race 2 (TO20, TO21, TO22, TO23, TO24, TO26); lanes *12–15*, sweet pepper pathotype (U48, Cns, Vdp3, 22210); lanes *16–19*, eggplant pathotype (Ibh, Y3-1, Chr208, Ara406)

map indicates that probes C and D should hybridize to a large band of at least 6 kbp. Thus, the source of the nonspecific faint band (around 2.5 kbp) observed in Fig. 6C and D, appears not to be a sequence in TomR1. Probes B and D showed some faint bands in isolates of eggplant pathotype (Fig. 6B, lane 12; Fig. 6D, lanes 10–12).

Pathogenicity test of Vdp4

To determine the race of isolate Vdp4 (tomato–sweet pepper pathotype), we examined its virulence on tomato cultivars (Fig. 7). Although TO20 (tomato pathotype race 2) was highly virulent on tomato cv. Momotaro carrying resistance gene *Ve*, Vdp4 was less virulent than race 1 isolate TV103. All three isolates were highly virulent on tomato cv. Oogatafukuju (Ve^{-}). Thus, Vdp4 was regarded as a race 1 isolate.

PCR using STS primer

On the basis of the results of genomic Southern hybridization (Figs. 5, 6), we concluded that the sequences of probe C (in TomR1, see Fig. 6) and B68-TV are specific to isolates pathogenic to tomato and to race 1, respectively. We designed PCR primers from the regions corresponding to probe C (Tr1: 5′-TGAAGTAGCCGATAGCTTTGT CTTGCCCGG-3′, Tr2: 5′-TGTCTGGATTAATCGCCG CAATAGAGACGC-3′) and B68-TV (Tm5: 5′-CGTA ACTTGAATCTCATTGACAGGAACAGG-3′, Tm7: 5′-GACTGCAGCTCGCGTCGCGAGCCCGACACC-3′) for pathotype-specific and race-specific amplification (Fig. 8). A PCR product (approximately 1.9 kbp) was specifically amplified in isolates pathogenic to tomato by primer pair Tm5/Tm7 designed from probe C. A PCR product (approximately 0.6 kbp) was specifically amplified in race 1 isolates

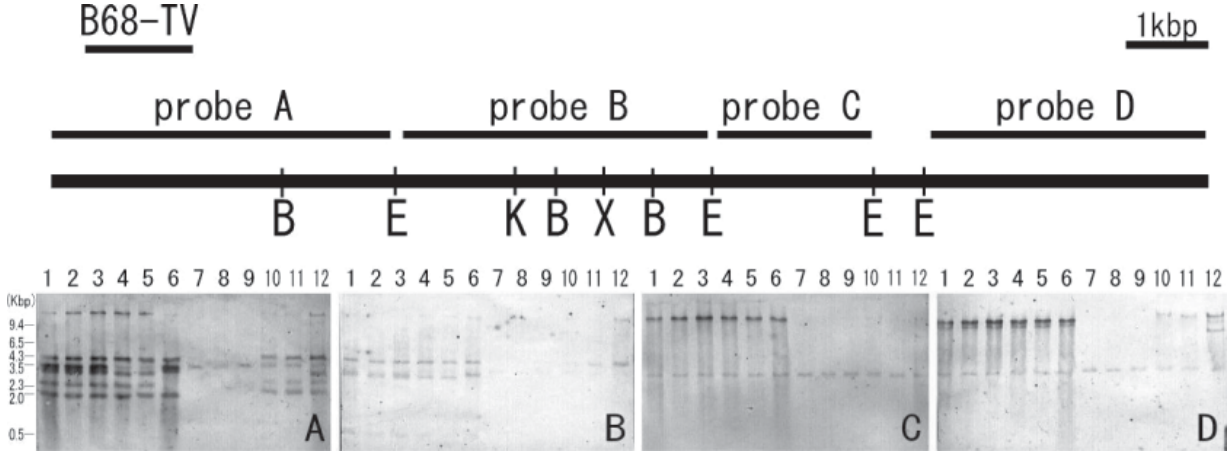

Fig. 6. Restriction map of TomR1 (*top*) and the results of genomic Southern blotting (*bottom*). Genomic DNAs of *Verticillium dahliae* were digested with *Bam*HI and hybridized with divided fragments of TomR1. Positions of hybridization probes (corresponding to the results of Southern blots, A–D) and RAPD product B68-TV are expressed in the map. *Letters* in the map are restriction sites: *B*, *Bam*HI; *E*, *Eco*RI; *K*, *Kpn*I; *X*, *Xba*I. Lanes *1–3*, tomato pathotype race 1 (TV103, U22, Shio); lanes *4–5*, tomato pathotype race 2 (TO20, TO26); lane *6*, tomato–sweet pepper pathotype (Vdp4); lanes *7–9*, sweet pepper pathotype (U48, Cns, Vdp3); lanes *10–12*, eggplant pathotype (Y3-1, Ibh, Chr208)

pathogenic to tomato by primer pair Tr1/Tr2 designed from B68-TV.

Discussion

Verticillium dahliae has been reported to be distinguishable from other closely related species, e.g., *Verticillium albo-atrum* and *Verticillium longisporum*, by RAPD analysis (Li et al. 1999; Messner et al. 1996; Zeise and Tiedemann 2002). Pérez-Artés et al. (2000) differentiated the cotton-defoliating and nondefoliating pathotypes of *V. dahliae* by RAPD, and Mercado-Blanco et al. (2001, 2002) sequentially designed PCR primers specific to each pathotype. These primers were used to quantitatively monitor the fungus during colonization of the host plant by real-time PCR (Mercado-Blanco et al. 2003). Japanese isolates of *V. dahliae* were analyzed by Koike et al. (1996). Although they were able to differentiate the tomato pathotype from other pathotypes by RAPD patterns, sweet pepper and eggplant pathotypes were indistinguishable from each other.

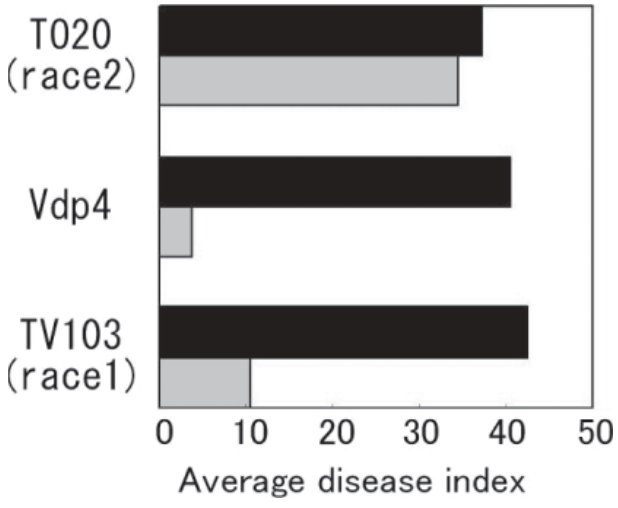

Fig. 7. Pathogenicity test on tomato cvs. *Filled columns*, Oogata-fukuju (Ve^-); *shaded columns*, Momotaro (Ve^+). Roots of nine plants were dipped into a spore suspension of *Verticillium dahliae* TV103 (tomato pathotype race 1), TO20 (tomato pathotype race 2), and Vdp4 (tomato–sweet pepper pathotype). Calculation of disease index for each plant and average index is described in Materials and methods

This study obtained RAPD-PCR products specific to pathotype and race of *V. dahliae*. E10-U48, amplified with primer E10, was specific to the isolates pathogenic to sweet pepper. In the genomic Southern analysis probed with E10-U48, even the tomato–sweet pepper and sweet pepper pathotypes were mutually distinguishable by band size. B68-TV amplified with primer B68 was specific to tomato pathotype race 1 and Vdp4 (a tomato–sweet pepper pathotype). Because the pathogenicity test in this study proved that Vdp4 was race 1, we conclude that B68-TV is specific to race 1 of isolates pathogenic to tomato. Furthermore, the genomic DNA fragment TomR1, which was cloned as the peripheral region of B68-TV, includes a sequence specific to all isolates pathogenic to tomato (tomato pathotype race 1, race 2, and the tomato–sweet pepper pathotype). Interestingly, Radišek et al. (2004) reported that high sequence similarity existed between a part of TomR1 and an amplified fragment length polymorphisms marker (AFLP-11) of *V. albo-atrum* pathogenic to hop. The meaning of this similarity remains unclear; further investigation about the character and function of TomR1 is necessary.

Our present and past studies identified PCR primers that allow specific amplification in tomato pathotype (Fig. 1C–E; Usami and Amemiya 2005; Usami et al. 2002, 2005). Although Vdp4 is highly virulent on tomato, these primers do not yield the same amplifications as the tomato pathotype from this isolate. Consistently, Koike et al. (1996) and Komatsu et al. (2001) dealt with Vdp4 as having the genotype similar to sweet pepper and eggplant pathotype. However, a primer pair (Tm5/Tm7) allowed certain identification of isolates pathogenic to tomato (including Vdp4) in our PCR assays. Races of these isolates were distinguishable by primer pair Tr1/Tr2. Although designing primers from E10-U48 is work for the future, all four pathotypes (tomato, tomato–sweet pepper, sweet pepper, and eggplant) and

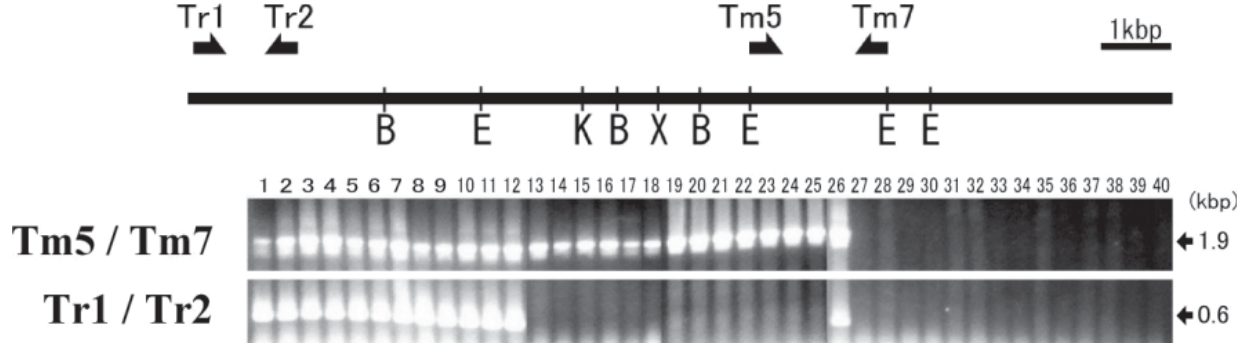

Fig. 8. Two pairs of sequence tagged site (STS) primers were designed from sequences for TomR1. Annealing sites of STS primers are expressed in a restriction map of TomR1 (*top*). PCR products amplified from genomic DNAs of *Verticillium dahliae* by primer pairs Tm5/Tm7 and Tr1/Tr2 were electrophoresed on agarose gel (*bottom*). *Letters* in the map are restriction sites: *B*, *Bam*HI; *E*, *Eco*RI; *K*, *Kpn*I; *X*, *Xba*I. Lanes *1–12*, tomato pathotype race 1 (TV103, U22, Shio, TO2, TK23, Kgm, Gto2, 84007, G-39, GM1, TR-1, MIH004); lanes *13–25*, tomato pathotype race 2 (Gto1, TO20, TO21, TO22, TO23, TO24, TO26, TK15, Gok1, Yam1, Hok1, Gca1, Vng); lane *26*, tomato–sweet pepper pathotype race 1 (Vdp4); lanes *27–36*, sweet pepper pathotype (U48, Cns, 22210, Vdp3, U20, P2-1, P2-2, P8-1, P9-1, P9-2); lanes *37–40*, eggplant pathotype (Y3-1, Chr208, Ibh, Ara406)

races might be distinguishable using the sequences obtained in this study.

Carder and Barbara (1994) reported that many Japanese isolates differed genetically from foreign isolates. In contrast, Koike et al. (1997) reported that Japanese isolates of tomato pathotype have a genetic relation with foreign RFLP group "A" proposed by Okoli et al. (1993). Because the genetic relation between Japanese and foreign isolates is unclear, investigating the effectiveness of our probes and primers for foreign isolates is very important future work. Currently, we believe that our primers can be used to identify and specifically detect Japanese isolates of *V. dahliae* for research and practical purposes. Each pathotype of *V. dahliae* has many common hosts, such as eggplant (Table 1), strawberry (Sakai et al. 1999), and cabbage (Sakai et al. 2001) in Japan. Thus, rapid identification of the pathotype is very important for the control of this pathogen. We could determine that isolate MIH004 (from lettuce; Table 1) is tomato pathotype race 1 by PCR assay using our primers before doing an inoculation test. This indicates the potential of these primers.

DNA sequences that are involved in pathogenicity determination have potential as exact DNA markers to identify pathotype and race. However, none of the genes involved in the pathogenicity of *V. dahliae* have been reported. Kawchuk et al. (2001) have already identified two genes (*Ve*1 and *Ve*2) that exist on the *Ve* locus. They also revealed that these resistance genes encode cell surface-like receptor proteins. Although the race-determining mechanism in the tomato–*V. dahliae* interaction is not clear, a race-specific fungal factor that interacts with these receptor molecules must exist. For exact identification using molecular biology techniques, we must investigate the pathogenic mechanisms of *V. dahliae* as well as analyze the functions of DNA sequences in this study.

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