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FUNGAL DISEASES

Involvement of two different types of *Verticillium dahliae* in lettuce wilt in Ibaraki Prefecture, Japan

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Abstract Although wilt diseases of various plants by *Verticillium dahliae* occur worldwide, Verticillium wilt of lettuce is relatively new and rare. In 2009, this disease appeared suddenly in commercial fields in Ibaraki Prefecture, Japan. To investigate the cause of the disease, we characterized each lettuce isolate for pathogenicity, mating types, and genetic characteristics and found two types of *V. dahliae*. A population that included at least two types of *V. dahliae* was the putative infection source of the disease. Effective management systems against *V. dahliae* isolates of different races and having different host ranges are necessary.

Keywords *Verticillium dahliae* · Verticillium wilt · Lettuce · Host range · Race · Mating type

Lettuce (*Lactuca sativa* L.) fields throughout the world remained unaffected by *Verticillium dahliae* until the mid-1990s (Atallah et al. 2011). Thereafter, wilt disease of cultivated lettuce caused by this pathogen was observed first in the United States (Subbarao et al. 1997; Vallad et al. 2005) and subsequently in Italy (Garibaldi et al. 2007). In October 2009, this disease appeared suddenly in two commercial fields in two areas (Yuki and Bando, separated by ca. 30 km) in Ibaraki Prefecture, Japan (Kaneda et al. 2011). Although Verticillium wilt of lettuce caused by *V. tricorpus* had occurred previously in Japan (Kanto et al. 2003, 2005; Usami et al. 2011), no outbreak of the disease caused by *V. dahliae* had been observed on cultured lettuce. In the present study, we examined the pathogenicities, mating types, and other genetic characteristics of five *V. dahliae* isolates that are causal agents of lettuce wilt (Table 1) to investigate the cause of the sudden disease occurrence in the two areas of Ibaraki Prefecture. One isolate, 09100-1, was isolated from a diseased lettuce plant in a field of Yuki City. The other four isolates were isolated from three diseased lettuce plants in a field of Bando City. Isolates 09096-2B and 09096-3B were obtained from different parts of the same lettuce plant.

Japanese isolates of *V. dahliae* are often divided into several pathogenicity groups based on their host range (Hagiwara 1990; Horiuchi et al. 1990). This grouping is useful for discriminating among isolates and for risk management of field crops. For inoculation tests to ascertain the group for each lettuce isolate, a spore suspension (10^9 spores in 100 mL water) of each isolate was prepared using the method described by Usami et al. (2007, 2011), and nine seedlings of tomato (*Solanum lycopersicum*) cv. Oogata-fukuju, 9 of tomato cv. Momotaro, 7 of sweet pepper (*Capsicum annuum* var. *grossum*) cv. Ace, 4 of eggplant (*Solanum melongena*) cv. Senryo-nigo, and 8 of Chinese cabbage (*Brassica rapa* var. *glabra*) cv. Taibyo-rokujunichi and of lettuce (*Lactuca sativa*) cv. Cisco were inoculated by root dipping. Inoculated and uninoculated

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Table 1 *Verticillium dahliae* isolated from diseased lettuce in Ibaraki Prefecture

Isolate	Geographic origin	Pathogenicity group[a,b]	Race[b,c]	*Ave1*[b,d]	Mating type[b]
09100-1	Yuki	Eggplant strain (group A)		−	*MAT1-1*
09095-1-2B	Bando	Tomato strain (group B)	1	+	*MAT1-2*
09096-2B	Bando	Tomato strain (group B)	1	+	*MAT1-2*
09096-3B	Bando	Tomato strain (group B)	1	+	*MAT1-2*
09095-4B	Bando	Tomato strain (group B)	1	+	*MAT1-2*

[a] Proposed by Hagiwara (1990) and Horiuchi et al. (1990)

[b] Investigated in this study

[c] On tomato cultivars

[d] In PCR assay of genomic DNA to detect sequence of avirulence gene *Ave1* in *Verticillium dahliae* corresponding to tomato resistance gene *Ve* (de Jonge et al. 2012)

+, detected; −, not detected

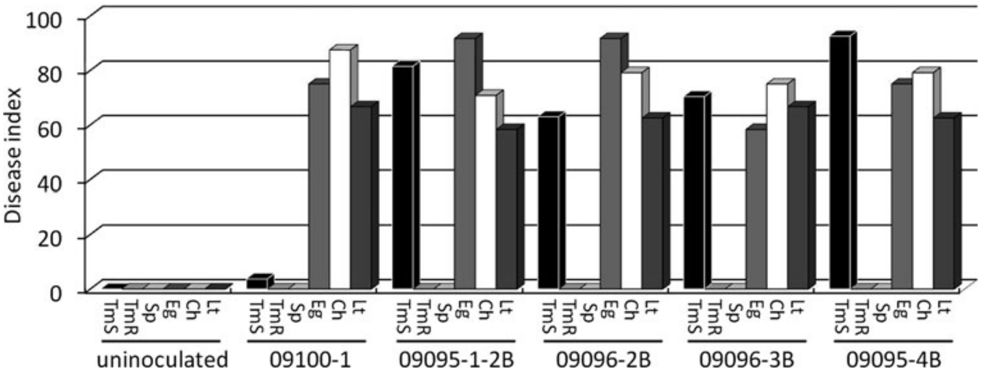

Fig. 1 Disease severity indices for tomato cvs. Oogata-fukuju (*TmS*) and Momotaro (*TmR*), sweet pepper (*Sp*), eggplant (*Eg*), Chinese cabbage (*Ch*), and lettuce (*Lt*) inoculated with each lettuce isolate of *Verticillium dahliae* from Ibaraki Prefecture. Indices of uninoculated controls are shown at *left*

plants were maintained in a growth chamber at 25 °C and a 12-h photoperiod for 1 month. The severity of external symptoms (wilting and yellowing) on plants were rated on a scale of 0–3, where 0, no symptoms; 1, symptoms on lower leaves; 2, on middle leaves; and 3, on upper leaves. The disease index was calculated using the formula [total symptom score/(3 × number of plants)] × 100.

Disease indices of the inoculation tests are presented in Fig. 1. Vascular discoloration was observed on diseased plants, and *V. dahliae* was re-isolated from them. In the tests, four isolates (09095-1-2B, 09096-2B, 09096-3B, and 09095-4B) produced clear symptoms on tomato cv. Oogata-fukuju and on eggplant with no symptoms on sweet pepper, indicating that these isolates are the tomato strain (group B). However, only a slight yellowing of just one lower leaf was observed on tomato cv. Oogata-fukuju inoculated with 09100-1. Growth suppression of tomato cv. Oogata-fukuju inoculated with this isolate was not observed, in contrast to the other four isolates (Fig. S1). Therefore, we concluded that isolate 09100-1 is nonpathogenic on tomato and on sweet pepper, indicating that this isolate is an eggplant strain (group A). All lettuce isolates were strongly pathogenic on Chinese cabbage as well as on lettuce, irrespective of the pathogenicity group (Fig. 1). Typical external symptoms of tomato cv. Oogata-fukuju and Chinese cabbage, and vascular browning of Chinese cabbage are presented in Fig. S1 of Electronic Supplementary Materials.

We used two tomato cultivars in pathogenicity tests (Fig. 1): cv. Oogata-fukuju, carrying no resistance gene, and cv. Momotaro, carrying resistance gene *Ve*. Isolates of *V. dahliae* that were pathogenic on tomato were divided into race 1 and race 2. Race 2 can overcome tomato cultivars carrying resistance gene *Ve*, although race 1 cannot (Alexander 1962). Four isolates that were pathogenic on tomato cv. Oogata-fukuju (carrying no *Ve*) caused no symptoms on cv. Momotaro (carrying *Ve*) (Fig. 1). This result indicates that these isolates are race 1 on tomato.

The relation of races and cultivars has been reported for lettuce as well. Although lettuce cultivars resistant to race 1 isolates were reported in the United States (Hayes et al. 2007; Vallad et al. 2006), race 2 isolates are still virulent on them (Vallad et al. 2006). Recently, the races of *V. dahliae* on tomato and lettuce were reported as correlated (Maruthachalam et al. 2010). Furthermore, Hayes et al. (2011) reported that the resistance gene of lettuce (*Vr1*) possibly corresponds to a gene that has sequence similarity with the resistance gene of tomato, *Ve*. According to these reports, the race of *V. dahliae* on lettuce is probably decided with the same avirulence factor as that on tomato. Therefore, 09095-1-2B, 09096-2B, 09096-3B, and 09095-4B are probably race 1 on lettuce cultivars.

We were unable to conduct inoculation tests because lettuce cultivars carrying a resistance gene were not available in Japan, so it should be done in the future. (The lettuce cv. Cisco, which we used in the pathogenicity tests, carries no resistance gene.) We were unable to conduct a race assessment of 09100-1 using tomato cultivars because this isolate is nonpathogenic on tomato. However, de Jonge et al. (2012) uncovered a sequence of *Ave1* that is an avirulence gene of *V. dahliae* on resistant tomato cultivar. Consequently, we were able to perform PCR assay to infer the race of 09100-1 on lettuce cultivars. We used PCR primers Ave1a (CTTCACTCTGCTCTCGTACAGA) and Ave1b (TTATATCTGTCTAAATTCGATGTTGACCGC) for the assay. The reaction program for amplification was 95 °C 5 min, (95 °C 30 s, 58 °C 30 s, 72 °C 30 s) × 25, 72 °C 5 min. In the PCR assay, the *Ave1* sequence was not amplified from genomic DNA of 09100-1 (Table 1). Therefore, it is likely that the isolate 09100-1 is race 2 on lettuce, suggesting that the lettuce cultivars carrying resistance gene *Vr1* would not be effective for this isolate even if the cultivars are introduced to Japan. In contrast, the *Ave1* sequence was amplified from genomic DNA of four isolates that had been determined as race 1 using inoculation tests (Table 1).

Although *V. dahliae* is an asexual fungus, it appears to be potentially heterothallic (Usami et al. 2009a, b). An isolate of *V. dahliae* has an idiomorph of mating type *MAT1-1* or *MAT1-2*, which includes mating-type-determining genes. The mating type of each lettuce isolate was examined by detecting a partial sequence of the idiomorph using PCR and genomic Southern blot hybridization. Detailed procedures of these assays were described in previous reports by Usami et al. (2009a, b). Results of genomic Southern hybridization probed with each mating type gene are presented in Fig. 2. These assays revealed that 09100-1 is *MAT1-1* and that the other isolates are *MAT1-2*. Usami et al. (2009b) reported that the mating type of *V. dahliae* is biased to *MAT1-2*; only five of 61 isolates were *MAT1-1*. In the present work, we found that 09100-1 is also an isolate of *MAT1-1*. Bias of the mating type indicates clonal propagation of *V. dahliae*. The origin of 09100-1 and the other four lettuce isolates are probably distinct.

In addition, genetic characteristics of respective isolate were examined using DNA markers VDf115 (Fig. S2a) and VDf130 (Fig. S2b) (Amemiya et al. 2000). Bands that are specific to isolates of tomato strain were detected when VDf115 and VDf130 were used as probes of genomic Southern hybridization in an earlier study (Amemiya et al. 2000). In the results presented here, the band pattern of four isolates of tomato strain (Fig. S2, lanes 2–5) was distinct from that of 09100-1, an isolate of eggplant strain (Fig. S2, lane 1). This result is consistent with the previous report (Amemiya et al. 2000) and supports the results of our pathogenicity tests.

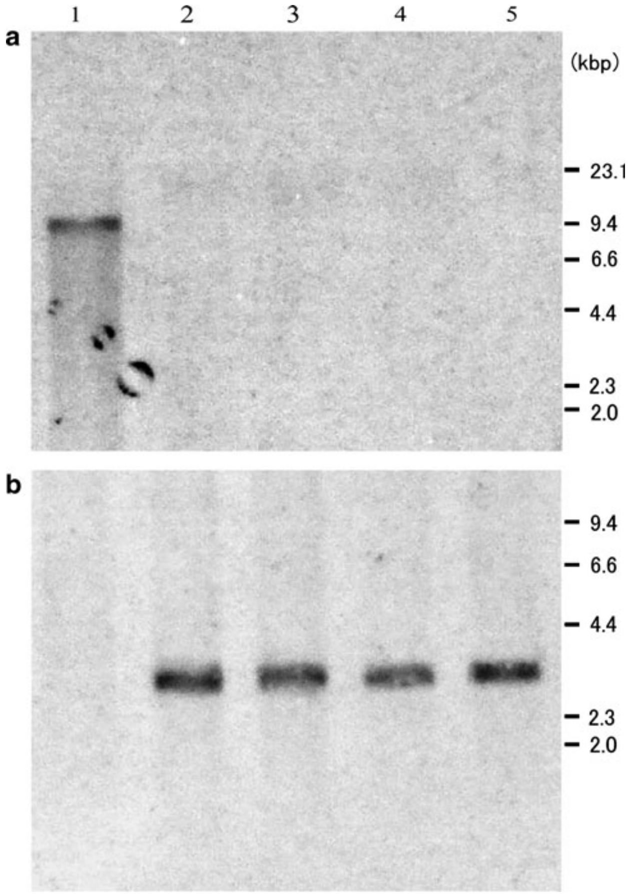

Fig. 2 Genomic Southern hybridization probed with *MAT1-1-1* (**a**) and *MAT1-2-1* (**b**) genes of *Verticillium dahliae*. *Lanes 1* 09100-1, *2* 09095-1-2B, *3* 09096-2B, *4* 09096-3B, and *5* 09095-4B. Fungal genomic DNA was digested with *Hin*dIII. Procedures for this assay follow Usami et al. (2009a, b)

All results obtained in this study indicate that 09100-1 and other four isolates differ distinctly in their characteristics (Table 1). The existence of different types of isolates precludes the possibility that the sudden occurrence of lettuce wilt by *V. dahliae* in Ibaraki Prefecture is due to a mutation that changed the pathogenicity. It is improbable that two isolates mutated simultaneously and acquired pathogenicity on lettuce. A population that includes these different types of *V. dahliae* is a possible inoculum source of the lettuce disease. Reportedly, the American lettuce isolates varied in their host ranges and race (Atallah et al. 2010; Qin et al. 2006; Vallad et al. 2006). Because *V. dahliae* can be transmitted on lettuce seed (Atallah et al. 2011; Vallad et al. 2005) and the United States is a principal lettuce-seed producing country for Japan, the disease in Ibaraki may have originated from seeds produced in the

United States. Although in a previous study, we found only three *MAT1-1* isolates among 49 Japanese isolates of *V. dahliae* (Usami et al. 2009b), in the present study, we found one of five lettuce isolates was *MAT1-1*, a rather high ratio of *MAT1-1* among the lettuce isolates. Atallah et al. (2010) reported that the *MAT1-1* ratio in the *V. dahliae* population in coastal California, a major lettuce-seed producing area in the USA, is ca. 30 %; thus, the high *MAT1-1* ratio among the Ibaraki lettuce isolates is easy to explain if they originated in California.

Although the exact reason for the sudden occurrence of lettuce wilt caused by *V. dahliae* in the USA remains unclear, Atallah et al. (2010) concluded that the outbreak of spinach wilt caused by the same pathogen might be associated with it. Similarly, a fungal population comprising various types of isolates on some domestic crop plants could also be an inoculum source of lettuce in Ibaraki Prefecture. It is noteworthy that all lettuce isolates of Ibaraki Prefecture were pathogenic on Chinese cabbage, a major crop plant in Yuki, Bando, and surrounding areas. Verticillium wilt of Chinese cabbage is also a severe disease in these areas and may also provide an inoculum source for the lettuce disease. Although *V. longisporum* has been reported as the predominant agent of Verticillium wilt of Chinese cabbage in Ibaraki Prefecture and surrounding regions (Ikeda et al. 2011), *V. dahliae* is also regarded as a causal agent of Verticillium wilt on Chinese cabbage in Ibaraki Prefecture because we isolated *V. dahliae* from diseased Chinese cabbage in Ibaraki Prefecture. No conspicuous outbreak of the disease caused by *V. dahliae* was observed on crops other than Chinese cabbage in those regions. However, further investigation is necessary to verify the relation between the wilt pathogen of Chinese cabbage and that of lettuce. Moreover, attention must be given to transmission of the agent from diseased lettuce to other crops, including Chinese cabbage, in the surrounding fields. For greater understanding of the disease and its causal agents, we must examine the pathogenicity of Japanese isolates of *V. dahliae* that have been reported as pathogens of other crop plants to lettuce. Furthermore, effective management systems against *V. dahliae* isolates having different host ranges and belonging to different races must be established.

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