Further Evidence of *Mirafiori lettuce big-vein virus* but Not of *Lettuce big-vein associated virus* with Big-Vein Disease in Lettuce

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ABSTRACT

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Mirafiori lettuce big-vein virus (MLBVV) and *Lettuce big-vein associated virus* (LBVaV) are found in association with big-vein disease of lettuce. Discrimination between the two viruses is critical for elucidating the etiology of big-vein disease. Using specific antibodies to MLBVV and LBVaV for western blotting and exploiting differences between MLBVV and LBVaV in host reaction of cucumber and temperature dependence in lettuce, we separated the two viruses by transferring each virus from doubly infected lettuce plants to cucumber or lettuce plants. A virus-free fungal isolate was allowed to acquire the two viruses

individually or together. To confirm the separation, zoospores from MLBVV-, LBVaV-, and dually infected lettuce plants were used for serial inoculations of lettuce seedlings 12 successive times. Lettuce seedlings were infected at each transfer either with MLBVV alone, LBVaV alone, or both viruses together, depending on the virus carried by the vector. Lettuce seedlings infected with MLBVV alone developed the big-vein symptoms, while those infected with LBVaV alone developed no symptoms. In field surveys, MLBVV was consistently detected in lettuce plants from big-vein-affected fields, whereas LBVaV was detected in lettuce plants not only from big-vein-affected fields but also from big-vein-free fields. LBVaV occurred widely at high rates in winter-spring lettuce-growing regions irrespective of the presence of MLBVV and, hence, of the presence of the big-vein disease.

Big-vein disease of lettuce, first reported by Jagger and Chandler in the United States (7), occurs in major lettuce-growing areas in cool to temperate and, sometimes, subtropical regions, and has become an important problem in lettuce (*Lactuca sativa* L.) production during cooler periods of the year (2,5,10,24). In Japan, the big-vein disease was first reported in 1973 and has become a serious problem in several regions producing lettuce from winter to spring (6). The economic importance of the disease results from the unsightliness of the foliage, which reduces market value, and from delays in head formation and decreases in head size, which reduce the proportion of harvestable plants.

Big-vein disease of lettuce is a soilborne disease that is spread by the fungus *Oplidium virulentus* (a noncrucifer strain of *O. brassicae* sensu lato). Use of the name *O. virulentus* instead of *O. brassicae* has been proposed on the basis of recent biological and molecular analyses on *Oplidium* spp. (8,9,20).

The causal agent of the big-vein disease was postulated to be a root-infecting virus, but the viral agent remained unknown for more than half a century. In 1983, a rod-shaped virus was found in field-grown lettuce with big-vein symptoms (10). Because the rod-shaped virus was always associated with big-vein-affected lettuce, the virus was assumed to be the agent that caused the big-vein disease without confirmatory evidences that the virus alone actually induced the big-vein symptoms in lettuce (5,10,24). The rod-shaped virus was named *Lettuce big-vein virus* and classified

as the type species of the genus *Varicosavirus* at the International Committee on the Taxonomy of Viruses (ICTV) in 2000 (13).

A second virus, *Mirafiori lettuce virus* (MiLV) was also found in field-grown lettuce plants with big-vein symptoms. Lettuce plants developed big-vein symptoms after mechanical inoculation of sap from infected plants or partially purified preparations of MiLV (18). Lettuce plants infected with vector-transmitted MiLV also showed the typical symptoms (11). In the 8th ICTV report (23), MiLV was renamed *Mirafiori lettuce big-vein virus* (MLBVV) in the genus *Ophiovirus* by the Study Group of ICTV (it was erratically appeared as old name *Mirafiori lettuce virus*), and *Lettuce big-vein virus* was renamed *Lettuce big-vein associated virus* (LBVaV) (1). However, lettuce plants with big-vein symptoms, and serologically negative for MLBVV but positive for LBVaV were described in the report of a field experiment in Italy in 2003 (19).

Both MLBVV and LBVaV have almost always been detected in field-grown lettuce plants with big-vein symptoms (4,15-17,19). Infection of lettuce plants separately with MLBVV or LBVaV, to verify the putative association of each virus with big-vein disease, is essential for the clarification of the etiology of big-vein disease. MLBVV and LBVaV were isolated from doubly infected plants by mechanical transmission with low rates of infection (11,18). However, follow-up efforts for mechanical transmission of the viruses in our laboratory were unsuccessful.

In our preliminary inoculation tests using fungal vectors carrying both MLBVV and LBVaV, 16 plant species in eight families were transplanted into infested soil and then examined by western blotting. Two months after the transplanting, both MLBVV and LBVaV were detected in leaves of lettuce, garland chrysanthemum (*Chrysanthemum coronarium* L.), tomato (*Lycopersicon*

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esculentum Mill.), and eggplant (*Solanum melongena* L.), while neither virus was detected in eight plant species, such as radish (*Raphanus sativus* L.), spinach (*Spinacia oleracea* L.), and broad bean (*Vicia faba* L.). MLBVV alone caused systemic but symptomless infection of cucumber (*Cucumis sativus* L.), and LBVaV alone did so in onion (*Allium cepa* L.), Welsh onion (*A. fistulosum* L.), and Chinese chive (*A. tuberosum* Rottler.). MLBVV systemically infected cucumber plants, while LBVaV did not. Furthermore, LBVaV systemically infected lettuce plants at high temperature, such as 27°C, while MLBVV did not (12). We attempted to use this evidence to obtain fungal isolates carrying MLBVV or LBVaV alone from doubly infected lettuce plants with big-vein symptoms.

We report details of the relationships between the two viruses and big-vein disease. In order to obtain many lettuce samples infected with either MLBVV or LBVaV alone, we developed methods for isolating MLBVV and LBVaV from doubly infected lettuce plants, and for separately and consecutively transferring viruses to lettuce seedlings using zoospores of a fungal isolate. Furthermore, we are reporting extensive survey data on the occurrence of the two viruses in big-vein-affected fields and in fields with no record of big-vein disease in major winter-spring lettuce-producing regions in Japan. Our conclusion is that MLBVV is definitely responsible for big-vein disease in lettuce.

MATERIALS AND METHODS

Vectors. Four soil samples with lettuce root debris were collected from four grower's fields, where plants exhibited characteristic symptoms of big-vein disease at Toyohama, Kagawa; Kannonji, Kagawa; Nantan, Hyogo; and Hikigawa, Wakayama in Japan. The fungus *O. virulentus* was trapped on a lettuce plant (cv. Cisco) planted on each soil sample and maintained on oriental melon (*Cucumis melo* L. var. *makuwa*) or lettuce plants. Single-sporangial isolates, designated as isolates KZ-1, KA-1, HY-1, and WT-1, were derived from each bulk culture in oriental melon or lettuce plants for soil samples from four fields, as described earlier (8,9,20). A virus-free single-sporangial isolate (WOMs-3) obtained from a Welsh onion plant (15) was used in all acquisition trials as a recipient of isolated MLBVV or LBVaV, or both because of its wide host range and for uniformity of transmission experiments.

Fungal isolates were maintained on lettuce plants grown under nonsterile conditions on presterilized sand under relatively low light in a greenhouse at a constant temperature of 15 to 20°C. A zoospore inoculum from each fungal isolate was prepared by briefly washing roots free of sand with water and immersing the roots in tap water for 10 to 15 min. Fifty milliliters of a zoospore suspension (1 to 7×10^5 zoospores/ml) was applied to 20 to 30 7-day-old lettuce seedlings in a 200-ml container. These seedlings were checked for fungal infection 2 weeks after the inoculation and transplanted into pots as described above.

Separation of MLBVV and LBVaV from doubly infected lettuce plants. Lettuce plants infected with single-sporangial isolates from four soil samples were positive for MLBVV and LBVaV. Seedlings of cucumber (cv. Yoshinari) and lettuce were used for isolating each virus. MLBVV infects cucumber plants systemically at 18°C but LBVaV does not, while LBVaV systemically infects lettuce plants at 27°C but MLBVV does not (12). Exploiting the differences in host reactions and temperature dependence, we attempted to isolate MLBVV and LBVaV from doubly infected plants, and to obtain fungal isolates with MLBVV or LBVaV alone, or both viruses together.

To obtain a fungal isolate with MLBVV alone, we inoculated cucumber seedlings with zoospores from each of the four single-sporangial isolates. After growing cucumber seedlings at 18°C for 2 to 3 weeks, we tested the first or second youngest leaves for viral infection by western blotting. Plants that gave positive reac-

tion for MLBVV were excised to remove roots at below the cotyledon and allow development of new roots. Rooted cucumber plants were transplanted to sterilized sand, grown at 18°C for another week, and then inoculated with virus-free zoospores of isolate WOMs-3. Two to three weeks after the inoculation, zoospores were collected from cucumber roots and used as MLBVV inocula for infecting 7-day-old lettuce seedlings. Two months after the last inoculation, lettuce plants were tested for viral infection.

To obtain a fungal isolate with LBVaV alone, lettuce plants were inoculated with zoospores from each of the four single-sporangial isolates. Three to four weeks after the inoculation, at 27°C, lettuce leaves were tested for virus infection. Lettuce seedlings positive for LBVaV alone were excised to remove roots at their stems above soil, rooted, and exposed to virus-free zoospores of the isolate WOMs-3. Two to three weeks after inoculation, the zoospores were obtained and used as inocula for LBVaV alone.

To obtain a fungal isolate carrying both MLBVV and LBVaV, lettuce seedlings were inoculated at 18°C with zoospores from each of the four isolates. Zoospores with both viruses were obtained as described above.

Serial transmission of MLBVV and LBVaV by the vector. Twenty-four 7-day-old lettuce seedlings were inoculated with zoospores of WOMs-3 carrying MLBVV or LBVaV alone, or both viruses together from a single-sporangial isolate of KZ-1. Inoculated lettuce seedlings were grown at 18°C for 2 months and tested for viral infection and scored for symptoms. Zoospores were collected from two or three lettuce plants each that had been infected with MLBVV or LBVaV alone, or both together, and used for inoculation of three further groups of 24 lettuce seedlings. The transfer was repeated 12 times.

Detection of MLBVV and LBVaV by western blotting. The presence of MLBVV and LBVaV was examined by western blotting with polyclonal antibodies against MLBVV and a monoclonal antibody against LBVaV. Seven monoclonal antibodies against LBVaV were produced according to the standard procedure described previously (21). One monoclonal antibody (MAb-11-12C) was used for specific detection of LBVaV. The polyclonal antibody against MLBVV was supplied by H. J. Vetten (Biologische Bundesanstalt, Braunschweig, Germany). The western blotting assay was described previously (3).

Field surveys and collection of samples in winter-spring lettuce-producing regions. In 2003 and 2004, field surveys were performed and samples were collected from 126 lettuce fields at 19 major winter-spring lettuce-producing regions in Japan. Approximately 25 lettuce-leaf samples per field were collected at random (regardless of the presence or absence of symptoms) from 15 lettuce fields with records of big-vein disease and 34 fields without such records at six regions in which big-vein disease was a serious problem. Samples were also collected from 77 fields at 13 regions in which the occurrence of big-vein disease has not been known. A total of 2,951 samples from 126 lettuce fields were tested for MLBVV and LBVaV infection by western blotting.

RESULTS

Detection of MLBVV and LBVaV in lettuce plants by western blotting. Since western blotting for the detection of MLBVV and LBVaV has proved more reliable than enzyme-linked immunosorbent assay (ELISA), plant samples were tested for MLBVV and LBVaV by western blotting using specific antibodies. Samples from MLBVV- and LBVaV-infected lettuce plants yielded a single band of a protein of 48 kDa (Fig. 1). The polyclonal antibody against MLBVV gave positive reaction with MLBVV-infected lettuce extracts up to 10^{-5} dilution, while it gave no reaction with LBVaV-infected lettuce extracts even at 10^{-1} dilution. In the same way, the monoclonal antibody MAb-11-12C

gave positive reaction with LBVaV-infected lettuce extracts up to 5×10^{-3} , while it gave negative reaction with MLBVV-infected lettuce extracts even at 10^{-1} dilution (data not shown).

Separation of MLBVV and LBVaV from doubly infected lettuce plants. MLBVV and LBVaV differ in their host reactions and temperature dependence. Based on these differences, we isolated MLBVV and LBVaV from doubly infected lettuce plants with big-vein symptoms. More than half of cucumber seedlings inoculated with zoospores from each of the four fungal isolates were systemically infected with MLBVV alone and none were positive for LBVaV by western blotting (Table 1). In the same way, 22 of 122 inoculated lettuce seedlings grown at 27°C were infected with LBVaV alone, while two were doubly infected with MLBVV and LBVaV (Table 2). Similarly, by inoculation of lettuce seedlings with zoospores from each of the four fungal isolates at 18°C, lettuce seedlings doubly infected with MLBVV and LBVaV were obtained (data not shown). Thus, 12 isolates of WOs-3 carrying MLBVV or LBVaV alone, or both viruses were obtained as described above, and used for the consecutive transfer of the virus to lettuce plants.

Symptoms on lettuce plants inoculated with either MLBVV or LBVaV. The fungal isolates of WOs-3, carrying MLBVV or LBVaV alone, or both viruses together, were used to examine the expression of symptoms (Table 3). A total of 24 lettuce seedlings were inoculated with zoospores of WOs-3 that carried MLBVV alone for each of the four fungal isolates. In the inoculated seedlings, only MLBVV was detected by western blotting. Although 3 of 16 and 2 of 15 lettuce seedlings infected with MLBVV from the fungal isolates HY-1 and WT-1, respectively, did not show big-vein symptoms within 2 months after the inoculation, all other lettuce seedlings infected with MLBVV alone developed big-vein symptoms. Similarly, we inoculated lettuce seedlings with zoospores of WOs-3 that carried only LBVaV. Western blotting revealed that more than 95% of lettuce seedlings were

infected with LBVaV alone, and none of the lettuce seedlings were infected with MLBVV. None of the infected plants developed big-vein symptoms.

Repeated transfer to lettuce of MLBVV and LBVaV by the fungal vector. Since MLBVV and LBVaV have been associated with big-vein-diseased lettuce plants, it is possible that MLBVV and LBVaV might interact with each other or might be involved in the transmission of another virus, as between *Potato virus Y* and *Potato virus X* and between *Groundnut rosette virus* and *Groundnut rosette associated virus* (14,22). To elucidate possible interactions, we transferred fungal vectors carrying MLBVV or LBVaV alone, or both viruses together consecutively 12 times to fresh lettuce seedlings (Table 4). No characteristic features between singly and doubly infected lettuce plants were observed during the transfers in terms of symptom development and virus multiplication based on western blotting analysis. MLBVV was transmitted by fungal isolates from source plants infected with MLBVV alone or with both MLBVV and LBVaV. Lettuce seedlings infected with MLBVV showed big-vein symptoms. Similarly, LBVaV was transmitted from source plants with LBVaV alone or both viruses together. None of the lettuce seedlings infected with LBVaV alone developed symptoms. The consecutive transfers by the fungal vector showed that MLBVV and LBVaV had apparently been separated.

Prevalence of MLBVV and LBVaV in lettuce fields. To determine the relationship between the big-vein disease and the two viruses, we collected leaf samples in big-vein-affected and nonaffected lettuce fields. We always found MLBVV in lettuce

Fig. 1. Detection of *Mirafiori lettuce big-vein virus* (MLBVV) and *Lettuce big-vein associated virus* (LBVaV) in lettuce plants by western blotting analysis of healthy seedling (lane 1), seedlings infected with MLBVV alone (lane 2), seedlings infected with LBVaV alone (lane 3), and seedlings infected with both viruses (lane 4). The blots were probed with **A**, polyclonal antibodies against MLBVV and **B**, monoclonal antibody against LBVaV.

TABLE 1. Infection of cucumber seedlings with *Mirafiori lettuce big-vein virus* (MLBVV) after exposing to four single-sporangial isolates of *Oidium virulentus* carrying MLBVV and *Lettuce big-vein associated virus* (LBVaV)^a

Fungal isolate	Virus-infected/inoculated cucumber seedlings ^b			
	MLBVV	LBVaV	Both viruses	None
KZ-1	17/32	0/32	0/32	15/32
KA-1	9/16	0/16	0/16	7/16
HY-1	11/20	0/20	0/20	9/20
WT-1	18/25	0/25	0/25	7/25

^a Each cucumber seedling was inoculated with zoospores that carried MLBVV and LBVaV, and grown at 18°C for 2 to 3 weeks.

^b Cucumber seedlings were tested for MLBVV and LBVaV infection by western blotting.

TABLE 2. Infection of lettuce seedlings with *Lettuce big-vein associated virus* (LBVaV) by exposing to four single-sporangial isolates of *Oidium virulentus* carrying *Mirafiori lettuce big-vein virus* (MLBVV) and LBVaV at 27°C^a

Fungal isolate	Virus-infected/inoculated lettuce seedlings ^b			
	MLBVV	LBVaV	Both viruses	None
KZ-1	0/35	5/35	0/35	30/35
KA-1	0/19	4/19	0/19	15/19
HY-1	0/39	9/39	2/39	28/39
WT-1	0/29	4/29	0/29	25/29

^a Each lettuce seedling was inoculated with zoospores that carried MLBVV and LBVaV, and grown at 27°C for 3 to 4 weeks.

^b Lettuce seedlings were tested for MLBVV and LBVaV infection by western blotting.

TABLE 3. Inoculation of lettuce seedlings with the fungal isolate WOs-3 carrying *Mirafiori lettuce big-vein virus* (MLBVV) or *Lettuce big-vein associated virus* (LBVaV) alone, or both viruses together, transferred originally from four fungal isolates

Fungal isolate (source of virus)	Viruses transferred to WOs-3	Virus-infected/inoculated lettuce seedlings ^a		Lettuce seedlings with symptoms/inoculated
		MLBVV	LBVaV	
KZ-1	MLBVV	16/24	0/24	16/24
	LBVaV	0/24	24/24	0/24
	MLBVV+LBVaV	18/24	24/24	18/24
KA-1	MLBVV	8/24	0/24	8/24
	LBVaV	0/24	23/24	0/24
	MLBVV+LBVaV	12/24	24/24	12/24
HY-1	MLBVV	16/24	0/24	13/24
	LBVaV	0/24	22/24	0/24
	MLBVV+LBVaV	20/24	23/24	18/24
WT-1	MLBVV	15/24	0/24	13/24
	LBVaV	0/24	24/24	0/24
	MLBVV+LBVaV	22/24	24/24	22/24
Control ^b	None	0/24	0/24	0/24

^a Lettuce seedlings were tested for MLBVV and LBVaV infection by western blotting.

^b Lettuce seedlings were inoculated with virus-free zoospores of the isolate WOs-3.

plants from big-vein-affected fields, while we never found it in plants from big-vein-free fields (Table 5). Irrespective of the presence or absence of big-vein disease, LBVaV was found in lettuce plants from all of the 19 winter-spring lettuce-producing regions (Tables 5 and 6). MLBVV was not detected in 131 (33%) of 396 lettuce plants tested from big-vein-affected fields due to low incidence of the disease. The proportion of LBVaV-infected plants in big-vein-affected fields was 93% (368/396), and it was higher in big-vein-free fields, where it was 61% (1,116/1,835). Out of 2,951 winter-spring lettuce plants tested, more than 65% (1,981/2,951) were infected with LBVaV, and out of 126 lettuce fields surveyed, only two fields were free of LBVaV, indicating that LBVaV is prevalent in the winter-spring lettuce-producing regions surveyed in Japan, irrespective of the presence or absence of MLBVV.

DISCUSSION

When the rod-shaped *Lettuce big-vein virus* was found in lettuce plants with big-vein symptoms in 1983 (10), the virus was assumed to be the causal agent of the big-vein disease, without further evidences confirming that this virus alone actually caused the big-vein symptoms in lettuce plants. In 2000, a second virus, MLBVV, was found in lettuce plants with big-vein symptoms and was shown to be the real causal agent of the big-vein disease (19). The new finding provided a big impact on the etiology and control of big-vein disease.

Lot et al. (11) and Roggero et al. (19) reported that MLBVV could be transferred alone from doubly infected lettuce plants to seedlings by mechanical transmission. They also reported that lettuce seedlings could be mechanically infected with partially purified MLBVV from *Chenopodium quinoa*. In our laboratory tests, however, we were unable to infect lettuce seedlings mechanically with MLBVV. Separation of MLBVV from lettuce plants infected doubly with MLBVV and LBVaV by mechanical transmission is very difficult. Lot et al. (11) and Roggero et al. (19) did not clearly describe the symptoms of the source plants they used. In the present study, we developed a method for separating MLBVV and LBVaV from doubly infected lettuce plants with big-vein symptoms by exploiting differences in their host reactions and temperature dependence.

TABLE 4. Twelve serial transfers of *Mirafiori lettuce big-vein virus* (MLBVV) and *Lettuce big-vein associated virus* (LBVaV) from lettuce source plants infected with MLBVV or LBVaV alone, or both viruses together to lettuce seedlings by zoospores of the fungal isolate WOMS-3

Transfer	Viruses transferred to WOMS-3	Virus-infected/inoculated lettuce seedlings ^a		Lettuce seedlings with symptoms/inoculated
		MLBVV	LBVaV	
2nd	MLBVV	16/24	0/24	16/24
	LBVaV	0/24	24/24	0/24
	MLBVV+LBVaV	18/24	22/24	18/24
	None ^b	0/24	0/24	0/24
4th	MLBVV	15/24	0/24	14/24
	LBVaV	0/24	21/24	0/24
	MLBVV+LBVaV	24/24	24/24	24/24
	None	0/24	0/24	0/24
8th	MLBVV	10/24	0/24	10/24
	LBVaV	0/24	24/24	0/24
	MLBVV+LBVaV	20/24	24/24	20/24
	None	0/24	0/24	0/24
12th	MLBVV	18/24	0/24	18/24
	LBVaV	0/24	23/24	0/24
	MLBVV+LBVaV	14/24	22/24	14/24
	None	0/24	0/24	0/24

^a Lettuce seedlings were tested for MLBVV and LBVaV infection by western blotting.

^b Virus-free WOMS-3 was used as the control.

Since MLBVV systemically infected cucumber plants and LBVaV did not, we obtained a fungal isolate that carried MLBVV alone from cucumber plants infected with MLBVV alone. On the other hand, LBVaV systemically infected onion plants, but MLBVV did not. We attempted to obtain a fungal isolate with LBVaV alone from LBVaV-infected onion plants. However, it was very difficult to remove the underground parts from onion plants to eliminate the fungal isolate and to generate new roots. We exploited the observation that LBVaV systemically infects lettuce plants at a high temperature, such as 27°C, while MLBVV does not (12), and separated LBVaV from doubly infected lettuce plants. Using these separation methods, we obtained fungal isolates that carried MLBVV or LBVaV alone, or both viruses together from doubly infected lettuce plants with big-vein symptoms. In the consecutive transfers, all lettuce seedlings inoculated with a fungal isolate that carried MLBVV alone were negative for

TABLE 5. Field survey of the occurrence of *Mirafiori lettuce big-vein virus* (MLBVV) and *Lettuce big-vein associated virus* (LBVaV) in lettuce fields in six winter-spring lettuce-producing regions where big-vein disease was a serious problem

Location	Fields (no.)	Samples tested (no.)	Viruses detected ^a			
			MLBVV	LBVaV	Both viruses	None
Fields with big-vein disease ^b						
Kamogawa, Chiba	1	25	0	2	23	0
Awajisima, Hyougo	5	125	0	44	76	5
Okayama, Okayama	1	36	0	24	9	3
Tuchinari, Tokushima	2	60	0	14	34	12
Kannonji, Kagawa	5	125	1	7	115	2
Itoman, Okinawa	1	25	0	13	7	5
Total	15	396	1	104	264	27
Fields without big-vein disease						
Kamogawa, Chiba	9	86	0	70	0	16
Awajisima, Hyougo	5	125	0	96	0	29
Okayama, Okayama	5	134	0	85	0	49
Tuchinari, Tokushima	4	100	0	88	0	12
Kannonji, Kagawa	6	150	0	106	0	44
Itoman, Okinawa	5	125	0	52	0	73
Total	34	720	0	497	0	0

^a Lettuce plants were tested for MLBVV and LBVaV infection by western blotting.

^b Lettuce samples were collected randomly in the indicated fields.

TABLE 6. Field survey of the occurrence of *Mirafiori lettuce big-vein virus* (MLBVV) and *Lettuce big-vein associated virus* (LBVaV) in lettuce fields in 13 winter-spring lettuce-producing regions with no record of big-vein disease

Location	Fields (no.)	Samples tested (no.)	Viruses detected ^a			
			MLBVV	LBVaV	Both viruses	None
Yuuki, Ibaraki	5	125	0	45	0	80
Iwai, Ibaraki	5	125	0	30	0	95
Komoro, Nagano	9	135	0	82	0	53
Syuuchi, Shizuoka	4	100	0	83	0	17
Ogasa, Shizuoka	3	75	0	55	0	20
Kakogawa, Hyogo	4	100	0	43	0	57
Zentsuji, Kagawa	3	75	0	28	0	47
Iyo, Ehime	6	150	0	71	0	79
Kitano, Fukuoka	7	175	0	78	0	97
Buzen, Fukuoka	5	125	0	110	0	15
Amakusa, Kumamoto	10	250	0	233	0	17
Shiraishi, Saga	5	125	0	63	0	62
Minamikushiyama, Nagasaki	11	275	0	195	0	80
Total	77	1,835	0	1,116	0	719

^a Lettuce plants were tested for MLBVV and LBVaV infection by western blotting.

LBVaV. All the MLBVV-infected lettuce plants developed big-vein symptoms. During serial transfers of LBVaV, all lettuce seedlings inoculated with LBVaV alone were negative for MLBVV and showed no symptoms, indicating that there was no contamination by MLBVV during the transfers. No specific features indicating interactions between the two viruses were observed in doubly infected plants. These results clearly indicated that MLBVV was the causal agent of the big-vein disease. The two viruses do not appear to coexist in an obligatory manner in lettuce and are probably transmitted independently from plant to plant by the fungus.

In earlier field surveys, almost all lettuce plants with big-vein symptoms contained both MLBVV and LBVaV (4,15–17,19). In our survey in major winter-spring lettuce-producing regions in Japan, lettuce plants were infected with LBVaV at high rates, regardless of the occurrence of big-vein disease. Since LBVaV is prevalent in winter-spring lettuce-producing regions, LBVaV-infected lettuce plants have a good chance of also being infected with MLBVV. Indeed, LBVaV was frequently but inconsistently found in lettuce plants with big-vein symptoms (5). The wider distribution of LBVaV can be partially explained by the fact that LBVaV infection progresses more rapidly than MLBVV infection (16) and LBVaV proliferates over a wider range of temperatures than MLBVV (12).

The results of our transmission tests with the fungal vector in the laboratory and our survey of lettuce fields clearly demonstrate that MLBVV is the causal agent of the big-vein disease and that MLBVV alone induces big-vein symptoms in lettuce plants. Observations of MLBVV virions under the electron microscope after negative staining are more difficult than LBVaV virions. Thus, LBVaV might be mistakenly regarded as the causal agent of the big-vein disease. Roggero et al. (19) found lettuce plants that showed big-vein symptoms and were serologically negative for MLBVV but positive for LBVaV. It is possible that the ELISA tests they used were not sensitive enough to detect low concentrations of MLBVV at later stages of infection. The identification of MLBVV as the causal agent of big-vein disease may facilitate control measures for the disease, for example, by objective evaluation of resistant cultivars, and monitoring and avoidance of growing lettuce in big-vein-affected fields.

Although the etiology of big-vein disease can only and unequivocally be established when infectious clones of MLBVV become available, based on our present studies, LBVaV is not specifically involved in the big-vein disease.

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