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VIRAL AND VIROID DISEASES

Lisianthus enation leaf curl virus, a begomovirus new to Japan, is more virulent than the prevalent tomato yellow leaf curl virus in *Ty*-gene-mediated resistant tomato cultivars

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Abstract
In 2020, tomato plants showing yellow leaf curl disease (TYLCD) were tested for begomovirus infection in Okinawa Prefecture, Japan. Amplification and sequence analysis of circular DNA in the diseased samples showed that some of the tomato plants carrying the *Ty-3a* resistance gene to tomato yellow leaf curl virus (TYLCV) were co-infected with TYLCV and lisianthus enation leaf curl virus (LELCV). LELCV infection was also detected in pepper plants in Okinawa by PCR, suggesting that the virus is widespread in the region. To characterize the interactions of LELCV, TYLCV-IL and TYLCV-Mld with tomato cultivars that carry different *Ty* resistance genes, we agroinoculated tomato plants with the viruses. The resistance conferred by *Ty-2* was effective against TYLCV-IL but not against TYLCV-Mld and LELCV. In contrast, *Ty-3a*-conferred resistance was effective against TYLCV strains but not fully effective against LELCV. Mixed infection with TYLCV-IL and LELCV in tomato plants bearing *Ty-3a* induced even more severe TYLCD symptoms and growth inhibition than did LELCV alone. Our study demonstrated that LELCV is a more virulent begomovirus species than the prevalent TYLCV isolates in resistant tomato cultivars.

Keywords Begomovirus · Disease control and pest management · Geminivirus · Lisianthus enation leaf curl virus (LELCV) · Tomato yellow leaf curl virus (TYLCV) · *Ty* resistance gene

Introduction

The genus *Begomovirus* comprises 445 species, more than any of the other six genera of the family *Geminiviridae* (ICTV 2021). The single-stranded DNA (ssDNA) genomes of begomoviruses are encapsulated in twinned-icosahedral particles, which are transmitted from plant to plant by an insect vector whitefly (*Bemisia tabaci*). Tomato yellow leaf curl disease (TYLCD), caused by some begomoviral species, is one of the most devastating viral diseases of tomato (*Solanum lycopersicum* L.) plants worldwide (Moriones and Navas-Castillo 2000). Tomato yellow leaf curl virus (TYLCV), a monopartite begomovirus, causes TYLCD in tomato and in at least 49 other species belonging to 16 families (Papayiannis et al. 2011). The global dissemination of the Israel (IL) and mild (Mld) strains of TYLCV started in the 1980s (Mabvakure et al. 2016).

TYLCD symptoms comprise more or less prominent upward curling of leaf margins, a reduction in leaflet area, yellowing of young leaves, flower abortion, plant stunting, and reduced yields (Koeda et al. 2015). If susceptible plants are infected during early growth, yields can be entirely lost (Cohen and Harpaz 1964). The use of cultivars with resistance loci *Ty-1* to *Ty-6*, identified from wild relatives of tomato, has proven to be an effective control strategy against TYLCD (Agrama and Scott 2006; Anbinder et al. 2009; Hutton and Scott 2014; Ji et al. 2009a, b; Zamir et al. 1994). The introgression of allelic loci *Ty-1*/*Ty-3*/*Ty-3a* or locus *Ty-2* into commercial tomato cultivars has been the major focus of resistance breeding globally, including in Japan (Saito et al. 2008; Yan et al. 2018).

In the long archipelago that comprises Japan, the first report of TYLCD caused by TYLCV was from Shizuoka and Aichi prefectures in the Tokai district in central

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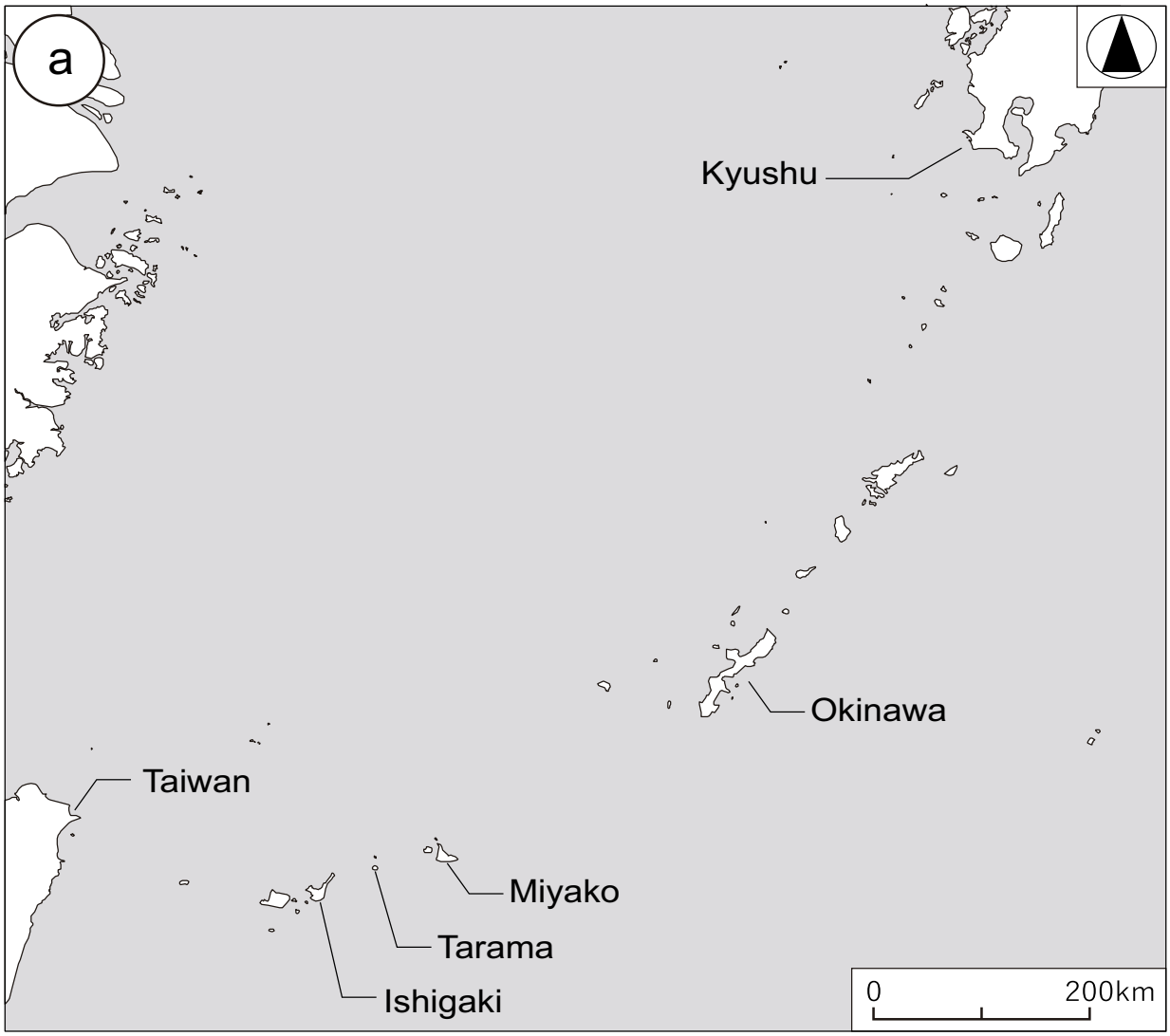
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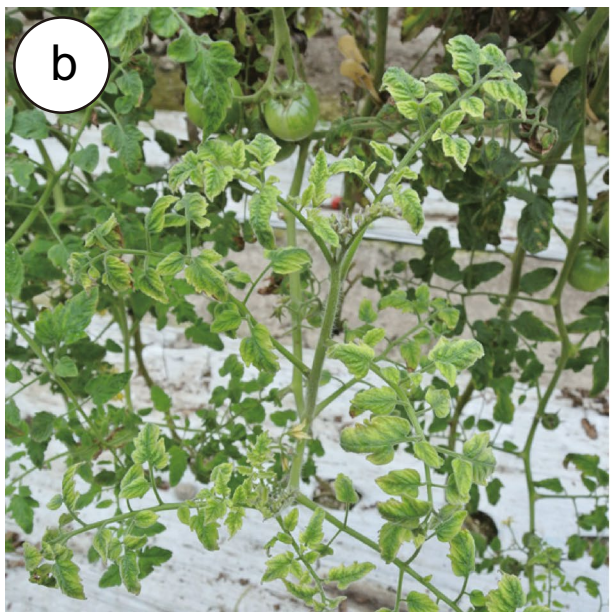
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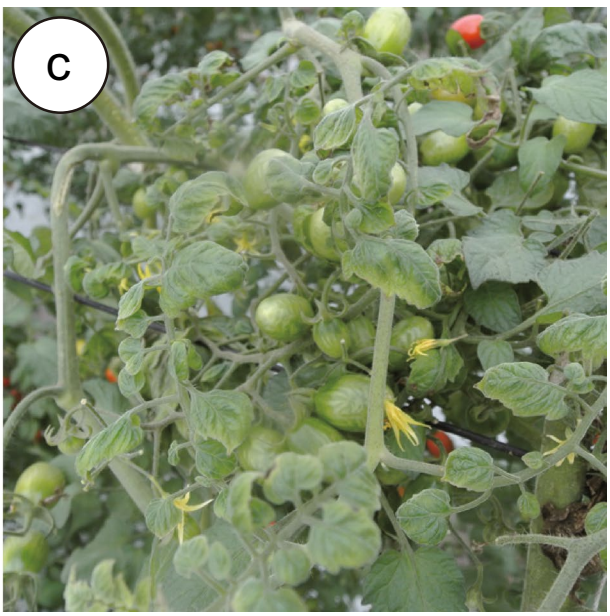
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◂**Fig. 1** Location of field surveys and symptoms of tomato yellow leaf curl disease (TYLCD) on tomato. **a** Location of islands in Okinawa Prefecture. TYLCD symptoms were observed on **b** a resistant tomato plant with the *Ty-3a* gene at Tomigusuku City, Okinawa Island and **c** a susceptible tomato plant in Itoman City

Japan and Nagasaki Prefecture in the Kyushu district in southwestern Japan in 1996 (Kato et al. 1998). Additional full-length genome sequences of TYLCV-Mld strain were isolated in Shizuoka, Aichi, and Mie prefectures in the Tokai district and Osaka Prefecture in the Kansai district, whereas sequences of TYLCV-IL strain were isolated from Nagasaki and Miyazaki prefectures of the Kyushu district and Kochi Prefecture of the Shikoku district (Koeda et al. 2015; Onuki et al. 2004; Ueda et al. 2004, 2005). Thus, TYLCV-IL isolates are mainly in western Japan, and TYLCV-Mld isolates are primarily in central and eastern Japan. Okinawa Prefecture constitutes a chain of 160 islands at the southwestern tip of the Japanese archipelago and is surrounded by the warm Kuroshio current. The subtropical climate thus differs from the temperate climate of the main islands of Japan. On Okinawa Island, TYLCV-IL isolates were identified that genetically resemble isolates from Kochi Prefecture (Ueda et al. 2009). Moreover, isolates of the monopartite begomovirus ageratum yellow vein virus (AYVV) were identified from ageratum, tomato, and common bean plants on Ishigaki Island (Okinawa Prefecture), which is only 230 km from Taiwan (Andou et al. 2010; Tomitaka et al. 2020). Among Japanese prefectures, considering its geographic location, Okinawa Prefecture thus has the highest risk of invasion by various tomato-infecting begomovirus species from Southeast Asia.

In the present study, we observed typical yellowing symptoms on TYLCV-resistant tomato plants possessing *Ty-3a* in Okinawa Prefecture and isolated the monopartite begomovirus lisianthus elation leaf curl virus (LELCV) for the first time in Japan. This virus was first isolated from diseased lisianthus (*Eustoma grandiflorum* L.) in central Taiwan in 2015 (GenBank accessions LC091538 and LC091539). More recently, the full length of the genomes of LELCV isolates from tomato and pumpkin plants from Taiwan in 2016 and 2019 were sequenced. Although multiple sequences of LELCV have been deposited in GenBank, fundamental information on LELCV such as disease symptoms induced and host responses of *Ty*-gene harboring tomato plants has been lacking. In the present study, we inoculated tomato plants that have either the *Ty-2* or *Ty-3a* resistance gene with single or mixed inoculations of LELCV and TYLCV. This is the first study to characterize LELCV and evaluate the effectiveness of *Ty* resistance genes.

Materials and methods

Field sampling, PCR diagnosis, and isolation of full-length viral sequences

Naturally infected tomato (*S. lycopersicum*), pepper (*Capsicum annuum* L., *Capsicum frutescens* L., and *Capsicum baccatum* L.), eggplant (*Solanum melongena* L.), and common bean (*Phaseolus vulgaris* L.) plants with virus-like symptoms, such as typical leaf yellowing caused by begomovirus, were collected on Ishigaki, Tarama, Miyako, and Okinawa islands in March 2020 (Fig. 1a). DNA was extracted from the collected samples using the Nucleon PhytoPure kit (Cytiva, MA, USA), and TYLCV-IL and TYLCV-Mld were initially detected by conventional PCR using TYLCV IL Mld uni F and R primers and the EmeraldAmp PCR Master Mix (Takara Bio, Kusatsu, Japan). PCR conditions were 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min; and 3 min final extension at 72 °C. The amplified products were separated by electrophoresis in a 1.0% (w/v) agarose gel. The presence of *Ty-2* and *Ty-3*/*Ty-3a* resistance genes in the collected tomato plants was confirmed using molecular markers and the method of Koeda et al. (2020).

The full-length nucleotide sequence of the genomes of the begomoviruses infecting susceptible and resistant tomato plants were amplified using a rolling-circle amplification (RCA)-based TempliPhi DNA Amplification Kit (Cytiva) as described by Koeda et al. (2015). Amplified products were cut into monomers using the restriction enzyme PstI for LELCV and BamHI for TYLCV and cloned for sequencing, and the sequences were phylogenetically analyzed as reported by Koeda et al. (2021).

Based on the sequences obtained in this study and from GenBank, a specific primer pair was designed for each begomovirus species and strain: LELCV F and R primers, TYLCV-IL F and R primers, and TYLCV-Mld F and R primers (Supplementary Table S1). The DNA polymerase KOD Plus neo (Toyobo, Osaka, Japan) was used for the PCR reaction according to the manufacturer's instructions. PCR conditions were 94 °C for 2 min; 35 cycles of 98 °C for 10 s, 64 °C for 30 s, and 68 °C for 1 min; 3 min final extension at 68 °C. The amplified PCR products were subjected to electrophoresis using a 1.0% (w/v) agarose gel. The PCR amplicons that indicated LELCV-positive samples were sequenced after treating with ExoSAP-IT Express (Thermo Fisher, Waltham, MA, USA). These obtained sequences were also phylogenetically analyzed.

Construction of infectious clones for isolates LELCV-[JR:Tom:T326], TYLCV-IL-[Jr:Ito:T278], and TYLCV-Mld-[JR:Tak]

Infectious clones for LELCV-[JR:Tom:T326] (LC642632), TYLCV-IL-[Jr:Ito:T278] (LC642631), and TYLCV-Mld-[JR:Tak] (AB921568) were obtained as reported by Koeda et al. (2017) using the primers listed in Supplementary Table S1. The obtained constructs were named pGreenII-p35S-LELCV, pGreenII-p35S-TYLCV-IL, and pGreenII-p35S-TYLCV-Mld.

Inoculation of tomato plants with begomoviruses

Plants of tomato cultivar Momotaro (M) (susceptible to begomovirus and a popular cultivar for fresh use in Japan) and resistant Momotaro Sakura (MS) (resistance genes *Ty-2*/*ty-2*) and Momotaro Peace (MP) (*Ty-3a*/*ty-3*) (Takii Seed, Kyoto, Japan), which share the Momotaro genetic background (Koeda et al. 2020) were grown in a growth room at 23 °C–30 °C with 13 h light/11 h dark.

Tomato plants were singly inoculated with LELCV, TYLCV-IL, or TYLCV-Mld using *Rhizobium radiobacter* (EHA105) transformed with pGreenII-p35S-LELCV, pGreenII-p35S-TYLCV-IL, or pGreenII-p35S-TYLCV-Mld using a colony inoculation procedure (Koeda et al. 2017). For mixed inoculations, tomato plants were first inoculated with TYCLV-IL, then with LELCV approximately 7 days after the first inoculation. After 20 or 35 days, plants were scored for disease severity as follows: 0, no symptoms; 1, very mild symptoms with slight leaf yellowing; 2, leaf yellowing; 3, leaves were small and yellowed; 4, leaves were small and distorted with yellowing. Plant height (cm) from the cotyledonary node to the shoot tip was measured. Mean disease severity index (DSI) score and mean plant height were analyzed for significant differences using the Bonferroni–Dunn test and the Tukey–Kramer test in Excel Toukei ver. 7.0 (Esumi, Tokyo, Japan), with $P < 0.05$ considered as statistically significant.

Quantification of virus titer by real-time PCR

Genomic DNA of LELCV, TYLCV-IL, and TYLCV-Mld was quantified by qPCR using the primer pairs in Supplementary Table S1 according to the methods of Koeda et al. (2021). The 25S ribosomal RNA gene from tomato genomic DNA was amplified using 25S-rRNA 2F and 2R (Koeda et al. 2020) for data normalization. Mean viral titers for LELCV and TYLCV were compared for significant differences using the Tukey–Kramer test in Excel Toukei ver. 7.0 (Esumi), with $P < 0.05$ considered as statistically significant.

Results

First isolation of monopartite begomovirus LELCV from Okinawa

From 18 of 28 tomato leaves with yellowing and other symptoms typical of begomoviruses collected in 2020 in Okinawa Prefecture, TYLCV was detected by PCR using the TYLCV IL Mld uni F and R primers and shown to be widely disseminated in Okinawa Prefecture (Table 1). When we genotyped these 28 symptomatic tomato plants for *Ty-2* and *Ty-3*/*Ty-3a* resistance loci using the molecular markers, 11 tomato plants were heterozygous for *Ty-3a* (Table 1).

A *Ty-3a*-bearing resistant tomato plant cultivated in Tomigusuku City in Okinawa Island had TYLCD symptoms and was positive for TYLCV infection (Fig. 1b). The RCA-amplified begomoviral full-length genomic sequence and pairwise comparisons clarified that the obtained sequence shared 98.9% similarity with LELCV-[BG9] (LC091539) isolated from lisianthus (*E. grandiflorum* L.) in Taiwan. The phylogenetic analysis revealed that LELCV sequences separated into three clusters: isolates from tomato and pumpkin plants (Cluster 1), isolates from tomato and lisianthus plants (Cluster 2), and isolates from tomato plants clustering with tomato yellow leaf curl Thailand virus (Cluster 3). LELCV-[JR:Tom:T326] (LC642632) isolated from Okinawa clustered with isolates from Taiwan in Cluster 2 (Fig. 2a). TYLCV-IL-[JR:Ito:T278] (LC642631), isolated from leaves of a susceptible tomato plant collected in Itoman City on Okinawa Island (Fig. 1c), had high similarity to the previously reported isolates from Okinawa and Kochi prefectures (Fig. 2b). TYLCV sequences isolated from Japan were in two clusters in the phylogenetic tree (Fig. 2b); cluster 1 comprised TYLCV-IL isolates from the western region, and cluster 2 comprised TYLCV-Mld isolates from the eastern region of Japan, including the Kansai district.

Because the TYLCV IL Mld uni F and R primers could not distinguish LELCV from TYLCV, we developed specific primers for LELCV, TYLCV-IL, and TYLCV-Mld based on our newly obtained sequences and those from GenBank. In the PCR of 171 leaf samples with viral-like symptoms from various horticultural crops, including tomatoes, peppers, eggplants, and common beans from 32 sites in Ishigaki, Tarama, Miyako, and Okinawa islands (Table 1), LELCV was detected in four tomato plants and 17 pepper plants from Ishigaki and Okinawa (Yaese, Itoman, Tomigusuku, and Nakagusuku) islands (Fig. 1a). TYLCV-IL was detected in tomato, pepper, eggplant, and common bean plants from four islands, but TYLCV-Mld was not detected in any of the samples from Okinawa Prefecture. Among 21 samples positive for LELCV, six were also infected with TYLCV-IL. Sequencing the PCR amplicons from these 21 samples

Table 1 Diagnosis of begomoviruses in horticultural crops in Okinawa Prefecture

Island	Locality	No. of fields	Species	No. of samples	Number of plants with				
					TYLCV[a]	TYLCV-IL[b]	TYLCV-Mld[b]	LELCV[b]	TYLCV-IL +LELCV[b]
Ishigaki	Ishigaki	4	Pepper (*Capsicum annuum*)	7	–	4	0	0	0
			Pepper (*Capsicum frutescens*)	11	–	9	0	0	1
Miyako	Miyako	7	Tomato (Susceptible)[c]	7	4	6	0	0	0
			Pepper (*Capsicum annuum*)	15	–	15	0	0	0
			Pepper (*Capsicum frutescens*)	8	–	3	0	0	0
			Eggplant	10	–	7	0	0	0
			Common bean	3	–	3	0	0	0
Tarama	Tarama	6	Pepper (*Capsicum annuum*)	3	–	3	0	0	0
			Pepper (*Capsicum frutescens*)	27	–	15	0	0	0
Okinawa	Yaese	1	Pepper (*Capsicum annuum*)	7	–	5	0	0	2
	Itoman	7	Tomato (Susceptible)[c]	4	3	3	0	1	0
			Pepper (*Capsicum frutescens*)	29	–	0	0	13	0
	Tomigusuku	2	Tomato (Resistance: *Ty-3a/ty-3*)[c]	10	9	7	0	0	3
	Nakagusuku	3	Tomato (Susceptible)[c]	6	2	6	0	0	0
			Tomato (Resistance: *Ty-3a/ty-3*)[c]	1	0	1	0	0	0
			Pepper (*Capsicum annuum*)	6	–	1	0	1	0
			Pepper (*Capsicum baccatum*)	6	–	1	0	0	0
	Uruma	2	Pepper (*Capsicum frutescens*)	11	–	0	0	0	0

[a]Virus infection was detected by PCR using TYLCV IL Mld uni F and R primers. Hyphen (-) indicates test was not done

[b]Virus infection was detected by PCR using virus-specific primers for TYLCV-IL, TYLCV-Mld, and LELCV

[c]The presence or absence of *Ty-2*, *Ty-3*, *Ty-3a* resistance genes was confirmed using molecular markers

showed that the 1087-bp partial sequences shared between 99.6 to 100% similarity with LELCV-[JR:Tom:T326] (Fig. 2c). These results thus revealed that TYLCV-IL and LELCV are the predominant begomoviruses infecting horticultural crops such as tomato plants in Okinawa.

LELCV induces symptoms in tomato plants harboring *Ty* genes

By 20 days post inoculation (dpi) with LELCV-[JR:Tom:T326] (LC642632), TYLCV-IL-[JR:Ito:T278] (LC642631), or TYLCV-Mld-[JR:Tak] (AB921568, from Osaka Prefecture; Koeda et al. 2015) of tomato cultivars M (susceptible), MS (*Ty-2/ty-2*), and MP (*Ty-3a/ty-3*) (Table 2), M plants infected with LELCV, TYLCV-IL, or TYLCV-Mld had typical TYLCD yellowing, curling, and stunting. MS plants infected with LELCV or TYLCV-Mld had symptoms similar to those on the M plants. In contrast, only 12–30% of the MS plants inoculated with TYLCV-IL were infected, and none of these infected MS plants had symptoms. All inoculated MP plants were infected with TYLCV-IL and TYLCV-Mld, but only some of the TYLCV-IL-infected plants had very slight yellowing, and none of the TYLCV-Mld-infected plants had symptoms. MP plants infected with LELCV had mild leaf yellowing and curling. Similar results were obtained in a second independent experiment (Table 2). These results indicate that *Ty-2*-conferred resistance is not effective against LELCV, and *Ty-3a*-conferred resistance is only partially effective.

When we used qPCR to assess the correlation between symptom expression and viral DNA accumulation using DNA from upper young leaves collected of the infected plants at 20 dpi (Fig. 3), significantly less TYLCV-IL viral DNA was present in MS (1/14) and MP (1/21) plants compared to M plants. In addition, the level of TYLCV-Mld viral DNA was significantly lower in MP (1/24) than in M and MS plants. Moreover, LELCV viral DNA accumulation was also significantly lower in MP (1/6) than in M and MS plants. Although LELCV viral DNA accumulation was lower in MP plants than in M plants (1/6), it was less restricted compared to TYLCV-IL (1/21) and TYLCV-Mld (1/24). Similar results were obtained in a second independent experiment. Overall, these results revealed that the repression of LELCV viral DNA accumulation conferred by *Ty-3a* in the heterozygous state is insufficient for MP to confer complete resistance.

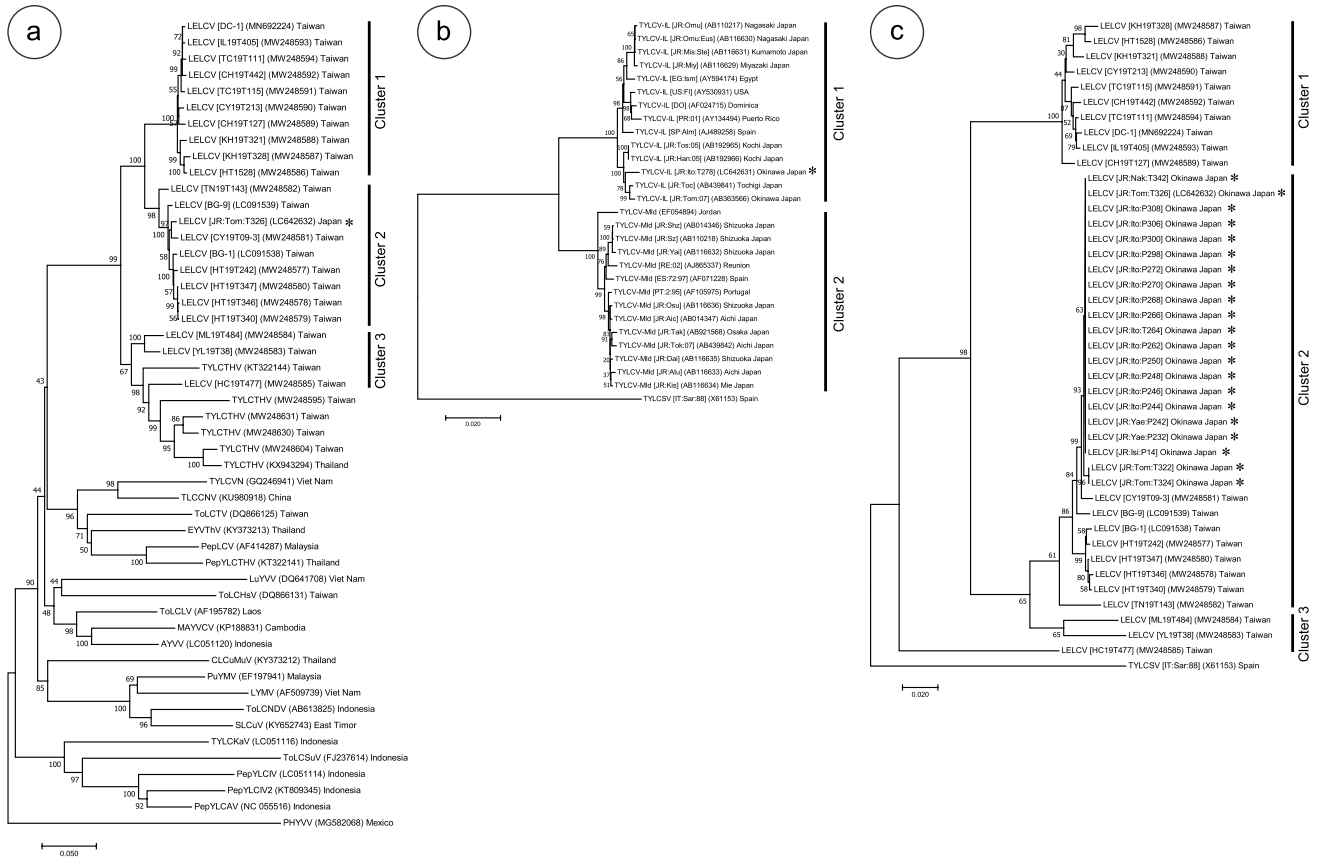

Fig. 2 Phylogenetic analysis of identified lisianthus enation leaf curl virus (LELCV) and tomato yellow leaf curl virus (TYLCV) isolates. **a** Neighbor-joining phylogenetic tree based on alignments of complete begomoviral sequences of LELCV with begomoviruses isolated from Southeast and East Asia, (**b**) on TYLCV, and (**c**) on partial sequences of LELCV. AYVV, Ageratum yellow vein virus; CLCuMuV, cotton leaf curl Multan virus; EYVThV, Emilia yellow vein Thailand virus; LYMV, Luffa yellow mosaic virus; LuYVV, Ludwigia yellow vein virus; MAYVCV, Malvastrum yellow vein Cambodia virus; PHYVV, pepper huasteco yellow vein virus; PepLCV, pepper leaf curl virus; PepYLCAV, pepper yellow leaf curl Aceh virus; PepYLCIV, pepper yellow leaf curl Indonesia virus; PepYLCIV2, pepper yellow leaf curl Indonesia virus 2; PepYLCTHV, pepper yellow leaf curl Thailand virus; PuYMV, pumpkin yellow mosaic virus; SLCCNV, squash leaf curl China virus; SLCuV, squash leaf curl virus; TLCCNV, tomato leaf curl China virus; ToLCHsV, tomato leaf curl Hsinchu virus; ToLCLV, tomato leaf curl Laos virus; ToLCNDV, tomato leaf curl New Delhi virus; ToLCSuV, tomato leaf curl Sulawesi virus; TYLCKaV, tomato yellow leaf curl Kanchanaburi virus; ToLCTV, tomato leaf curl Taiwan virus; TYLCTHV, tomato yellow leaf curl Thailand virus; TYLCVV, tomato leaf curl Vietnam virus; TYLCSV, tomato yellow leaf curl Sardinia virus. PHYVV (MG582068) or TYLCSV (X61153) was used as the outgroup. The begomovirus isolates sequenced in this study are indicated by an asterisk (*). Bootstrap percentages are given at the nodes (based on 1000 replicates). The branch lengths are proportional to the number of nucleotide changes, as indicated by the scale bar (0.05 substitution per site)

Mixed infection with LELCV and TYLCV-IL inhibits the growth of *Ty-3a*-bearing resistant tomato plants

Single-infected M (susceptible) and MS (*Ty-2*/*ty-2*) plants at 35 dpi had symptoms similar to those in the previous experiments, and mixed infection of TYLCV-IL and LELCV also induced severe symptoms on these plants (Fig. 4a, Table 3). In contrast, MP plants singly infected with TYLCV-IL or LELCV showed slight and mild symptoms, respectively, and the symptoms were more severe on the plants infected with both viruses.

The height of plants after the single and mixed infection with TYLCV-IL and LELCV at 35 dpi did not differ significantly among the mock-inoculated M, MS, and MP plants (Tukey–Kramer test) (Fig. 4b). However, the height of single- and mixed-infected M plants, LELCV single-infected and mixed-infected MS plants was significantly lower than that of the mock-inoculated plants. Similarly, the height of MP plants after single infections with TYLCV-IL or LELCV was significantly lower than that of the mock-inoculated plants, and the stunting was more severe after mixed infection with the two viruses. Similar results were obtained in a second and third independent experiments. These results showed that a single infection with LELCV inhibited the growth of *Ty-3a*-bearing resistant tomato plants and mixed infection with TYLCV-IL caused greater inhibition.

Table 2 Single agroinoculation of tomato with IL and Mld strain of tomato yellow leaf curl virus (TYLCV) and lisianthus enation leaf curl virus (LELCV)

Inoculum	Exp	Cultivar (resistance gene)[a]	Number of plants			(% of plants)[c]	DSI score[d]
			Inoculated	Infected[b]	No. symptomatic		
TYLCV-IL	1	M	5	5	5	100	3.6[a]
		MS (*Ty-2*)	10	3	0	0	0[b]
		MP (*Ty-3a*)	5	5	2	40	0.8[b]
	2	M	5	5	5	100	4.0[a]
		MS (*Ty-2*)	17	2	0	0	0[b]
		MP (*Ty-3a*)	5	5	0	0	0[b]
TYLCV-Mld	1	M	5	5	5	100	2.2[a]
		MS (*Ty-2*)	5	4	4	100	2.3[a]
		MP (*Ty-3a*)	5	5	0	0	0[b]
	2	M	5	4	4	100	2.0[a]
		MS (*Ty-2*)	5	5	5	100	2.0[a]
		MP (*Ty-3a*)	5	5	0	0	0[b]
LELCV	1	M	5	5	5	100	4.0[a]
		MS (*Ty-2*)	5	5	5	100	3.6[a]
		MP (*Ty-3a*)	5	5	5	100	1.0[b]
	2	M	5	5	5	100	4.0[a]
		MS (*Ty-2*)	5	5	5	100	3.8[a]
		MP (*Ty-3a*)	5	5	5	100	1.0[b]

[a]M = Momotaro (*Ty* negative, susceptible control), MS = Momotaro Sakura (*Ty-2*/*ty-2*), and MP = Momotaro Peace (*Ty-3a*/*ty-3*)

[b]Virus infection was detected by PCR

[c](Number of plants with symptoms ÷ Number of plants infected) × 100

[d]The disease severity index (DSI) ranged from 0 to 4: 0, no symptoms; 1, very mild symptoms with slight yellowing; 2, yellowing; 3, small leaves with yellowing; 4, small and distorted leaves with yellowing. Plants were surveyed for symptoms approximately 20 dpi. Different letters indicate a significant difference between means (Bonferroni–Dunn test, $P < 0.05$)

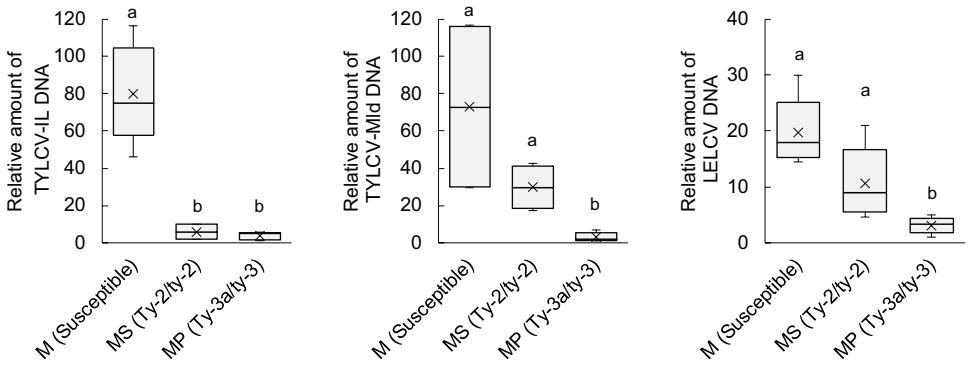

Fig. 3 Quantification of viral DNA in young, upper leaves of M, MS, and MP tomato plants 20 days post inoculation with either TYLCV and LELCV. M = Momotaro (*Ty*-negative, susceptible control), MS = Momotaro Sakura (*Ty-2*/*ty-2*), and MP = Momotaro Peace (*Ty-3a*/*ty-3*). Viral DNA values were normalized to the 25S rRNA gene. Within each box, the horizontal line is the median value (× is the mean), and the lower (and upper) edges of the box are the 25th (75th) percentile. Whiskers represent the upper and lower ranges. Different letters within a row indicate significant differences between the means (Tukey–Kramer test, $P < 0.05$)

In the relative quantification of begomoviral DNA extracted from upper young leaves collected at 35 dpi from single- and mixed-infected plants using qPCR (Fig. 4c) to examine the correlation between symptom expression and level of viral DNA, regardless of single or mixed infection, TYLCV-IL viral DNA accumulation was significantly lower in MS and MP plants than in M plants. In contrast, LELCV viral DNA accumulation was significantly lower in the MP

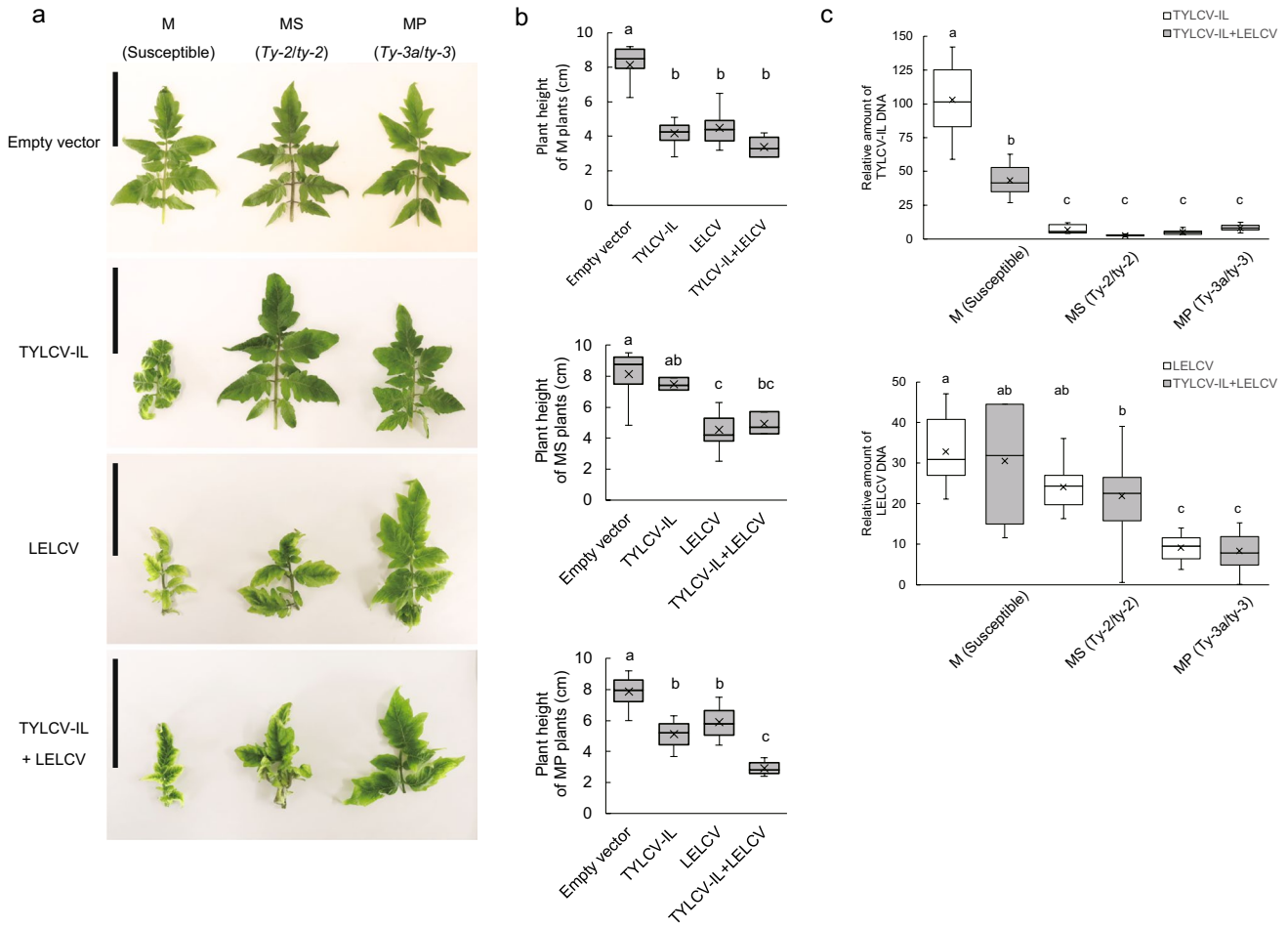

Fig. 4 Mixed infection of tomato M, MS, and MP plants with TYLCV and LELCV. **a** Leaves and **b** plant heights of uninfected, TYLCV-infected, LELCV-infected, and mixed-infected M, MS, and MP plants. Bars = 6 cm. **c** Level of TYLCV and LELCV DNA in young, upper of single- or mixed-infected M, MS, and MP plants 35 days post inoculation. M = Momotaro (*Ty* negative, susceptible control), MS = Momotaro Sakura (*Ty-2/ty-2*), and MP = Momotaro Peace (*Ty-3a/ty-3*). Viral DNA values were normalized to the 25S rRNA gene. Within each box, the horizontal line is the median value (× is the mean), and the lower (and upper) edges of the box are the 25th (75th) percentile. Whiskers represent the upper and lower ranges. Different letters within a row indicate significant differences between the means (Tukey–Kramer test, $P < 0.05$)

plants than in M and MS plants. However, LELCV levels did not differ significantly between LELCV-single-infected and mixed-infected MP plants. Overall, these results revealed that the repression of LELCV DNA levels conferred by *Ty-3a* in the heterozygous state is insufficient for complete resistance in MP.

Discussion

Since the first evidence of TYLCD caused by TYLCV in 1996 (Kato et al. 1998), the considerable efforts to introgress genetic resistance from wild relatives into tomato cultivars have met with success for controlling TYLCD in Japan. However, invasion by new begomovirus species has been a continuous threat as exemplified by our present isolation of LELCV for the first time in Japan in Okinawa Prefecture.

Because Okinawa Prefecture is located close to Taiwan and Southeast Asia, the infection by distinct begomovirus species from TYLCV is not surprising. The monopartite begomovirus AYVV is found in Southeast and East Asia, mainly in tropical and subtropical regions. AYVV has also been found on Ishigaki Island, most probably invading from Taiwan, and causes diseases in tomato, common bean, and ageratum plants (*Ageratum conyzoides* L.) (Andou et al. 2010; Tomitaka et al. 2020). We also frequently observed symptomatic ageratum plants around the greenhouse on Ishigaki Island during the field survey in the present study. Shahid et al. (2014) isolated AYVV from tomato plants cultivated in Tokyo, which has a high genetic similarity with the AYVV isolate from Ishigaki Island. However, since these

Table 3 Single and mixed agroinoculations of tomato plants with lisianthus enation leaf curl virus (LELCV) and tomato yellow leaf curl virus (TYLCV) IL strain

Inoculum	Exp	Cultivar[a]	Number of plants			(% of plants)[c]	DSI score[d]
			Inoculated	Infected[b]	No. symptomatic		
TYLCV-IL	1	M	10	9	9	100	4.0[a]
		MS (*Ty-2*)	10	3	0	0	0[b]
		MP (*Ty-3a*)	10	10	3	30	0.3[b]
	2	M	10	10	10	100	3.8[a]
		MS (*Ty-2*)	15	3	0	0	0[b]
		MP (*Ty-3a*)	10	10	4	40	0.4[b]
	3	M	10	10	10	100	3.7[a]
		MS (*Ty-2*)	20	4	0	0	0[b]
		MP (*Ty-3a*)	10	10	6	60	0.6[b]
LELCV	1	M	10	10	10	100	4.0[a]
		MS (*Ty-2*)	10	10	10	100	3.7[a]
		MP (*Ty-3a*)	10	10	7	70	0.7[b]
	2	M	10	9	9	100	4.0[a]
		MS (*Ty-2*)	15	15	15	100	3.8[a]
		MP (*Ty-3a*)	10	10	8	80	0.8[b]
	3	M	10	10	10	100	4.0[a]
		MS (*Ty-2*)	10	10	10	100	3.8[a]
		MP (*Ty-3a*)	10	9	9	100	0.8[b]
TYLCV-IL	1	M	10	10	10	100	4.0[a]
		MS (*Ty-2*)	10	7	7	100	4.0[a]
+LELCV		MP (*Ty-3a*)	10	10	10	100	1.5[b]
	2	M	10	10	10	100	4.0[a]
		MS (*Ty-2*)	20	3	3	100	4.0[a]
		MP (*Ty-3a*)	10	9	9	100	2.0[b]
	3	M	10	9	9	100	4.0[a]
		MS (*Ty-2*)	20	15	15	100	4.0[a]
		MP (*Ty-3a*)	20	19	19	100	1.9[b]

[a]M = Momotaro (*Ty* negative, susceptible control) and MP = Momotaro Peace (*Ty-3a*/*ty-3*)

[b]Virus infection was detected by PCR. In case of the mixed inoculated plants, infected means positive for both LELCV and TYLCV

[c](Number of plants with symptom ÷ number of plants infected) × 100

[d]The DSI ranged from a score of 0 to 4 as follows: 0, no symptoms; 1, very mild symptoms with slight yellowing; 2, yellowing; 3, small leaves with yellowing; 4, small and distorted leaves with yellowing. Symptom survey was conducted on approximately 35 dpi. Different letters indicate significant differences between means (Bonferroni–Dunn test, $P < 0.05$)

isolates were found in 2011, AYVV infection has not been reported on the main island of Japan, suggesting that AYVV is not widespread.

Twenty-one LELCV isolates from Taiwan are present in GenBank. Among these isolates, LELCV-[BG-9] (LC091539) from lisianthus (*E. grandiflorum*) had the highest similarity (98.9%) with LELCV-[JR:Tom:T326], which was isolated in Okinawa in the present study and clustered with isolates infecting tomato and lisianthus plants in the phylogenetic tree (Fig. 2a). Ueda et al. (2009) isolated full- and partial-length TYLCV-IL sequences from tomato plants cultivated at several sites on Okinawa Island in 2007. One full-length and six partial sequences of approximately 1400 bp were isolated from Tomigusuku, and an additional eight partial sequences from Uruma, Yomitan-son, and Nakijin-son. All these sequences were genetically similar to the TYLCV-IL isolate from the Kochi Prefecture in their phylogenetic analysis. These 14 partial TYLCV-IL sequences were not deposited in a database, so we could not compare them with the sequence of our LELCV isolate obtained from Tomigusuku in 2020, their phylogenetic analysis strongly suggests that LELCV was not present in tomato plants in 2007. The partial sequence of genomic DNA of LELCV isolates from the 21 samples collected in Okinawa Prefecture shared high similarity (99.6–100%) with that of LELCV-[JR:Tom:T326], indicating that genetic diversity of

LELCV from Okinawa is low compared to that from Taiwan (Fig. 2c). Based on the results of pairwise comparisons and phylogenetic analyses and considering the proximity of Okinawa to Taiwan, LELCV probably invaded Okinawa between 2007 and 2020 via an infected plant of lisianthus, tomato, or other species or via a viruliferous whitefly.

Here, we detected LELCV in tomato and pepper plants from Ishigaki and Okinawa islands, and TYLCV-IL was detected in tomato, pepper, eggplant, and common bean plants from Ishigaki, Tarama, Miyako, and Okinawa islands (Fig. 1a, Table 1). TYLCV can infect pepper and common bean plants (Kashina et al. 2003; Navas-Castillo et al. 1999; Quiñones et al. 2002; Roye et al. 1999; Salati et al. 2002). Morilla et al. (2005) reported symptomless infection of pepper plants by TYLCV-Mld and IL from Spain, and low frequency of transmission via whitefly from tomato to pepper plants, but not from pepper to tomato plants. Moreover, TYLCV-IL but not but TYLCV-Mld was transmitted by whiteflies to *C. annuum*, *C. chinense*, and *C. frutescens* (Polston et al. 2006). In Okinawa, *C. frutescens* plants are perennial, semidomesticated crops mainly grown in home gardens and small fields for cooking (Yamamoto and Nawata 2006). Because we detected LELCV and TYLCV-IL in *C. frutescens* plants (Table 1), these plants may act as reservoirs for the viruses. Further study is needed to clarify the infectivity and virulence of LELCV to pepper plants and transmission between pepper and tomato plants via whitefly. In addition, using the primer sets developed in this study to monitor LELCV presence in large tomato-producing areas on the main Japanese islands will be beneficial.

Although LELCV sequences were isolated from Taiwan in 2015, 2016, and 2019, the virus has not yet been characterized. Here, we evaluated the effectiveness of the widely used *Ty-3a* and *Ty-2* resistance genes in tomato against TYLCV-IL, Mld, and LELCV (Figs. 3, 4; Tables 2, 3). As found in previous studies, *Ty-2* was fully effective against TYLCV-IL but was not effective against TYLCV-Mld and other monopartite and bipartite begomoviruses (Table 2) (Barbieri et al. 2010; Hanson et al. 2000; Ohnishi et al. 2016; Prasanna et al. 2015; Shahid et al. 2013; Tsai et al. 2011; Yamguchi et al. 2018). The *Ty-3a* resistance gene was effective against both the IL and Mld strains of TYLCV, but tomato plants occasionally developed a slight yellowing when infected with an IL strain. In contrast, *Ty-3a*-conferred resistance was not fully effective against LELCV, and MP plants developed mild symptoms. Moreover, symptoms and growth inhibition of MP plants became more severe after a mixed infection with LELCV and TYLCV-IL (Fig. 4a, b). The alleles *Ty-1*, *Ty-3*, and *Ty-3a* encode an RNA-dependent RNA polymerase, and *Ty-1* confers resistance to TYLCV by increasing cytosine methylation of viral genomes, suggesting enhanced transcriptional gene silencing (TGS) (Butterbach et al. 2014; Verlaan et al. 2013). However, begomoviruses are able to suppress the host antiviral silencing response (Vanitharani et al. 2005). Since single infection of MP with LELCV induced mild symptoms and growth inhibition was more severe after mixed infection (Fig. 4a and b), LELCV might have a suppressor protein against the TGS conferred by *Ty-3a*. However, the more severe symptom expression cannot be explained solely by the amount of viral DNA because there were no significant differences in the level of DNA of LELCV and TYLCV-IL between single and mixed infections (Fig. 4c). Shafiq et al. (2017) reported similar results for cotton (*Gossypium arboretum* L.); although disease severity on cotton plants was positively correlated with begomovirus DNA accumulation, some plants only had very mild or no symptoms despite having a relatively high virus titer, whereas other plants had severe symptoms but relatively low virus titers. Further functional characterization of open reading frames of LELCV is needed to clarify this point. Some of the begomovirus species from Southeast Asia are more virulent than TYLCV, and *Ty*-conferred resistance is also not fully effective against these viruses (Koeda et al. 2020). Therefore, from a practical point, evaluating and preparing pyramided commercial cultivars with multiple *Ty* resistance genes are imperative for tomato production for the Japanese tomato market.

In this study, we isolated LELCV from symptomatic *Ty-3a*-bearing resistant tomato plants in Okinawa for the first time in Japan and characterized its virulence in comparison with the prevalent TYLCV-IL and -Mld isolates. The emergence of multiple begomovirus species in tomato cultivation areas increases the chance of their recombination, a driving force of their evolution, extending their host range, and increasing their virulence (Kesumawati et al. 2019; Koeda et al. 2021, 2022; Lefeuvre and Moriones 2015). Moreover, several studies have inferred that the use of *Ty*-gene-harboring tomato cultivars may serve as a positive selection pressure on specific begomovirus species or recombinants (Belabess et al. 2015; García-Andrés et al. 2009). Further field study using a larger number of samples collected from multiple regions in Japan is required.

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Declarations

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

Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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