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Research Article

Survey of Passionfruit Viruses in Okinawa, Japan: Prevalence, Transmission, and Control of Passiflora Latent Virus Using Virus-Free Seedlings

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Abstract

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In Okinawa Prefecture, passionfruit cultivation during the winter and spring seasons has become problematic due to the emergence of symptoms, e.g., shriveled new leaves and reduced plant vigor. In 2017, a survey of 84 fields was conducted across the region. The symptoms of suspected virus diseases were confirmed, and the outbreaks of 6 specific viruses in Japan were further investigated via reverse transcription-polymerase chain reaction (RT-PCR). The results showed that the top three common symptoms were the curling of adult leaves, leaf surface cracking, and yellowing between leaf veins. Among the 6 viruses tested, East Asian Passiflora distortion virus (EAPDV), East Asian Passiflora virus-AO (EAPV-AO), and Passiflora latent virus (PLV) gave an incidence of 2.4%, 1.2%, and 85.7%, respectively, whereas Broad bean wilt virus-2 (BBWV-2), Cucumber mosaic virus (CMV), and EAPV-IB were not detected. Plants infected with EAPDV and EAPV-AO showed typical symptoms of leaf shriveling and fruit malformation, but those infected with PLV showed several other nonspecific symptoms. Based on the survey results, PLV was identified as the primary pathogen. The virus was isolated from infected passionfruit, causing symptoms in quinoa but not in passionfruit. PLV was also confirmed to be transmitted by insect vectors, e.g., cotton aphids, as well as by juice remaining on pruning shears. Furthermore, as PLV was detected in passionfruit seedlings obtained from a supplier, thus the supply of confirmed PLV-free seedlings was established as a countermeasure in Okinawa starting from 2019. Since then, the occurrence of PLV has dramatically decreased, as revealed in a second field survey conducted in 2022 showing a significantly lower number of PLV-infected fields. Abnormal symptoms of passionfruit and high incidence of PLV infection showed a strong positive correlation in Okinawa.

Key words: Aphid, Pruning shears, RT-PCR, DECS-C, *Carlavirus*.

INTRODUCTION

Passionfruit is a perennial plant belonging to genus *Passiflora*. There are approximately 400 known species in this genus, most of them originating from tropical and subtropical South America. Of these species, only approximately 50–60 are edible. The main passionfruit species cultivated in Japan is *Passiflora edulis* Sims, known as the purple species or native species. Passionfruit is grown in Kagoshima Prefecture, Okinawa Prefecture, and Tokyo, mainly on the Ogasawara

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Islands (Yonemoto & Kondo 2020). In Okinawa Prefecture, passionfruit cultivation began in the 1960s, and the cultivation area has been expanding each year. In recent years, however, abnormal virus-like symptoms, e.g., the shriveling of new leaves and a declined vigor, have been observed in passionfruit fields in Okinawa Prefecture during the winter/spring season. Passionfruit seedlings are propagated by cuttings, and if an infection is established via sap-sucking aphids or other vectoring insects, the seedlings continue to carry the virus. Therefore, there is a concern that the disease may be spread through propagated seedlings supplied by seed and plant companies.

In Japan, the outbreaks of five major viruses infecting passionfruit have been reported, i.e., Cucumber mosaic virus (CMV) (Yonaha *et al*. 1979), Broad bean wilt virus 2 (BBWV-2) (Yonaha *et al*. 1993; Kobayashi *et al*. 2004), Passiflora latent virus (PLV) (Watanabe *et al*. 1997), East Asian Passiflora virus (EAPV) (Iwai *et al*. 2006), and East Asian Passiflora distortion virus (EAPDV) (Riska *et al*. 2019). In 2020, Nakasato *et al*. (2020) reported the detection of two potyviruses, Uraria mosaic virus and Passiflora foetida virus Y (PfVY), in passionfruit in Japan. Globally, more than 20 viruses have been reported in plants of genus *Passiflora*, and it is possible that additional, yet-undiscovered, viral species exist.

In general, the identification of viruses as pathogens is based on Koch's postulates, which involve introducing the isolated viruses into healthy plants to reproduce the symptoms. However, viral diseases in passionfruit involve numerous factors, making it extremely complex to identify the pathogens. Specifically, disease development is influenced by the susceptibility of specific plant varieties to viruses, the virulence of the infecting virus/viruses, and environmental factors that promote symptom expression. For instance, PLV is known to show no or few signs of pathogenesis (Pares *et al*. 1997; Spiegel *et al*. 2007); conversely, symptoms have been reported to vary by origin of country. Although the cause is not clear, it is possible that different PLV strains exist. In addition, passionfruit has been reported to be infected by multiple viruses simultaneously. In Australia, passionfruit tip necrosis symptoms are thought to be caused by a mixed infection with passionfruit woody potyvirus (PWV) and CMV (Pares *et al*. 1985). Moreover, disease symptoms can differ from severe to minor, depending on the virus types. Among these, EAPV and EAPDV are known to negatively affect yields by causing fruit woodiness (Iwai *et al*. 2006; Riska *et al*. 2019). In particular, two EAPV types exist i.e., a fulminant form known as EAPV-AO which causes severe symptoms in passionfruit, and a mild form known as EAPV-IB. The abbreviations AO and IB stand for Amami Oshima and Ibusuki, respectively, indicating the regions where these strains were first identified.

There have been several cases of successful control of viral diseases in passionfruit. In Gifu Prefecture, Japan, a report indicated that the infection rate of CMV can be reduced by controlling aphids on passionfruit grown in the open air (Muramoto & Suzuki 2020). Another case involved the eradication of EAPV, where it was demonstrated that the virus could be controlled by removing old passionfruit seedlings and introducing healthy, virus-free seedlings. EAPV was first identified in 1986 on Amami Oshima Island, an isolated island in Kagoshima Prefecture. In 1992 and 1997, EAPV-AO was found to have spread across it as well (Iwai & Omatsu 2002). On Kikaijima, 30 km east of Amami Oshima Island, an isolated nursery of virus-free healthy seedlings was established, and cuttings propagated there were distributed to farmers to prevent the spread of the virus. As a result, a survey conducted from 2008 to 2010 revealed that EAPV-AO had significantly decreased to a few cases only in some areas of the island (Fukumoto *et al*. 2012; Iwai 2017).

In the present study, we investigated the cause of abnormal symptoms in passionfruit grown in Okinawa Prefecture from winter to spring by testing samples for a variety of previously reported viral diseases in Japan. Because the outbreak survey confirmed the high frequency of viral infections, we also examined the ef-

fect of suppressing the occurrence of abnormal symptoms by introducing virus-free seedlings.

MATERIALS AND METHODS

Sampling and observation of disease symptoms in the field

In the 2017 survey, leaf sampling was conducted from January to August in passionfruit fields on the two main islands of Okinawa, Miyako Island, and Ishigaki Island, mainly targeted plants with suspected viral symptoms. A total of 133 samples were collected from 84 fields.

Of these 133 samples, 104 samples were evaluated for field symptom observation and classification and the result was presented in Table 1 and Fig. 1. The remaining 29 samples- including 10 from Miyako Island, 16 from Ishigaki Island, and 3 from the southern region of Okinawa's main island- were excluded from symptom evaluation due to the absence of detailed field observation at those sites.

The plants sampled on the main island of Okinawa were classified according to the following symptoms (Fig. 1): A, leaf curling; B1, new shoot stunting; B2, leaf stunting; C, cracked leaf surface; D1, leaf yellowing; D2, yellowing between leaf veins; E, reduced vigor; and F, fruit malformation. The sampled leaves were placed in plastic bags and stored in the freezer at -80℃ until reverse transcription-polymerase chain reaction (RT-PCR) was performed.

In the 2022 survey, sampling was conducted in June in the southern part of the main island of Okinawa with a total of 58 samples collected from 21 fields.

In both surveys, virus detection was conducted solely by RT-PCR, and no virus isolation was performed from the field samples.

RNA extraction and RT-PCR analysis

The collected samples were subjected to RNA extraction for virus identification. Total RNA was isolated from both the young and old leaves of each plant using ISOGEN, reagent for RNA extraction (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's protocol. In brief, 1 mL of ISOGEN was added to 0.05 g of sample and grind. The solution was transferred into a 1.5-mL tube, added with 200 µL of chloroform, and shaken for 1 min. It was then centrifuged at 12,000 rpm (model 3780; Kubota, Tokyo, Japan) at 2℃ for 10 min,

Table 1. Identification of virus-like symptoms in passionfruit fields in Okinawa, Japan in 2017.[z]

	No. of virus-like symptoms[y]							
Survey location	A	B1	B2	C	D1	D2	E	F
The northern area of the main island	17	12	0	15	5	9	6	0
Motobu Town	2	1	0	1	0	0	0	0
Onna Village	15	11	0	14	5	9	6	0
The central area of the main island	1	1	3	0	0	0	0	3
Nishihara Town	1	0	0	0	0	0	0	0
Uruma City	0	1	3	0	0	0	0	3
The southern area of the main island	32	6	2	11	8	13	0	0
Itoman City	30	6	2	11	8	13	0	0
Nanjo City	1	0	0	0	0	0	0	0
Yaese Town	1	0	0	0	0	0	0	0
Total No.	50	19	5	26	13	22	6	3

[z] Symptom classification in Table 1 is based on visual observations of 104 passionfruit plants. Since multiple symptoms were often observed on a single plant, the total number of symptom entries amounts to 144.

[y] A: leaf curling; B1: new shoot stunting; B2: leaf stunting; C: leaf surface cracking; D1: yellowing of leaves; D2: yellowing between leaf veins; E: reduced vigor; and F: fruit malformation.

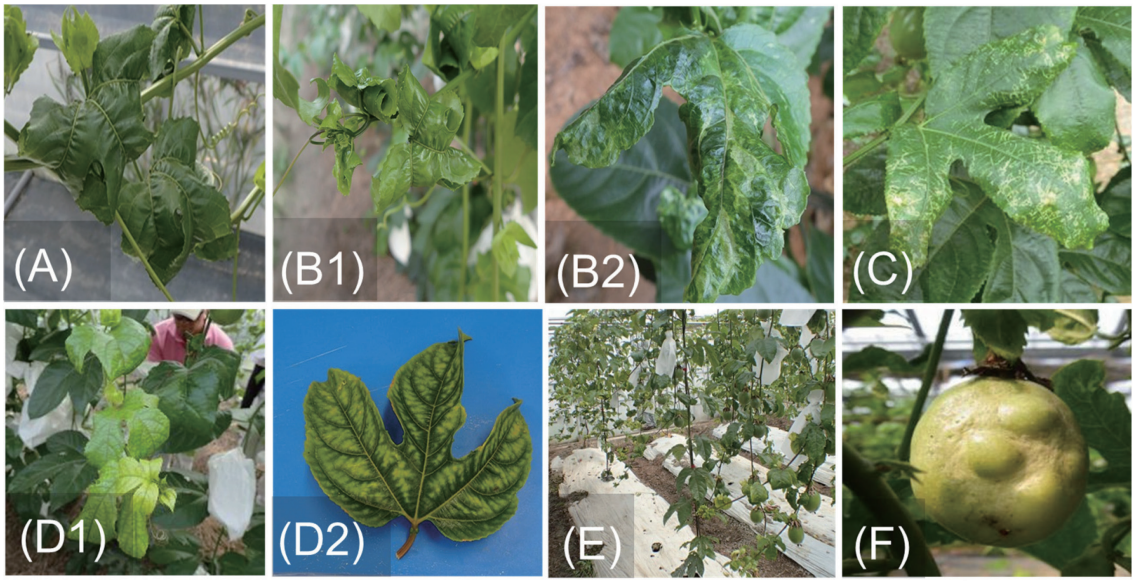

Fig. 1. Virus-like symptoms identified in passionfruit fields in Okinawa Prefecture, Japan: (A) leaf curling; (B1) new shoot stunting; (B2) leaf stunting; (C) leaf surface cracking; (D1) yellowing of leaves; (D2) yellowing between leaf veins; (E) reduced vigor; and (F) fruit malformation.

and 400 μL of the supernatant was transferred into a 1.5-mL tube, added with 400 μL of isopropanol, and shaken for 1 min. After centrifugation at 12,000 rpm at 2℃ for 10 min, the liquid in the 1.5-mL tube was discarded and 800 μL of 75% ethanol was added to dislodge the precipitate accumulated at the bottom by tapping the tube. After a second centrifugation at 12,000 rpm at 2℃ for 10 min, all the liquid in the 1.5-mL tube was carefully removed using a pipette, ensuring that the precipitate remained on the bottom. The tube was then inverted and left standing for 15 min to allow the evaporation of any remaining ethanol. Finally, 100 μL of RNase-free H_2O was added and the pellet was gently resuspended by tapping the tube to ensure complete dissolution. The obtained RNA was used as the template for reverse transcription reactions.

The presence of 6 viruses (BBWV-2, CMV, EAPDV, EAPV-AO, EAPV-IB, and PLV) was assessed via RT-PCR using previously published primers (Kondo *et al*. 2005; Tang *et al*. 2008; Fukumoto *et al*. 2012; Okada *et al*. 2017; Riska *et al*. 2019) which are listed in Table 2.

For the detection of EAPDV, cDNA was synthesized using the PrimeScript II 1st strand cDNA Synthesis kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The oligo(dT) primers listed in Table 2 were used for reverse transcription reactions. SapphireAmp Fast PCR Master Mix (Takara Bio, Shiga, Japan) was used for polymerase chain reaction (PCR), and reactions were carried out under the following conditions: one pre-denaturation cycle at 94℃ for 5 min; denaturation at 94℃ for 1 min; annealing at 55℃ for 1 min; extension at 72℃ for 2 min for 30 cycles in sets; and one extension cycle at 72℃ for 10 min.

For the other 5 viruses (BBWV-2, CMV, EAPV-AO, EAPV-IB, and PLV), RT-PCR amplifications were performed using the PrimeScript™ One Step RT-PCR Kit Ver. 2 (Dye Plus) (Takara Bio, Shiga, Japan). The thermal cycling conditions were as follows: pre-denaturation at 94℃ for 2 min, 25 cycles of denaturation for 30 s at 94℃, primer annealing for 30 s at 55℃, extension for 1 min at 72℃, and final extension at 72℃ for 10 min.

All PCR products were electrophoresed

Table 2. Primers used in this study for reverse transcription-polymerase chain reaction (RT-PCR) amplifications.

Target virus[z]	Name	Sequence 5′-3′	Size	Reference
BBWV-2	BBWVKMRM	TDGWDCCATCVAGICKCATTTT	320 bp	Kondo *et al*. (2005)
	BBWVVSSP	GTBTCDAGTGCTYTDGAAGG		
CMV	mul-CM-F2	GTTGCTTCCTGATTCAGTCAC	446 bp	Okada *et al*. (2017)
	mul-CM-R2	ACTACCAACTCAGCTCCCG		
EAPDV	oligo(dT)	CTAACTGTGGAAAGTCTTCC	800 bp	Riska *et al*. (2019)
	PV-F5	ACTCAATGCTGGGGACGCTC		
	PV-R7	CCCATACCAAGTAATGTGTG		
EAPV-AO	EAPVAO-F	TGCATGTCCTAGACCTC	1,201 bp	Fukumoto *et al*. (2012)
	AOIB200	T_{16}AGGACAAC		
EAPV-IB	EAPVIB-200	GACAAGAACGCCAGTTTG	1,245 bp	Fukumoto *et al*. (2012)
	AOIB200	T_{16}AGGACAAC		
PLV	PLV-F	CGAGACACACGCAAACGAA	523 bp	Tang *et al*. (2008)
	PLV-R	CAGCAAAGCAAAGACACGA		

[z] BBWV-2: Broad bean wilt virus-2; CMV: Cucumber mosaic virus; EAPDV: East Asian Passiflora distortion virus; EAPV-AO: East Asian Passiflora virus-AO; EAPV-IB: East Asian Passiflora virus-IB; PLV: Passiflora latent virus.

on a 1.5% agarose gel with GelRed (Biotium, Fremont, CA, USA) and irradiated with UV to confirm DNA amplification.

Identification of PLV in passionfruit using the DECS-C method

Total RNA was first extracted from 500 mg of leaves from a stunted passionfruit plant obtained from a seedling company (sampled in 2019). For comprehensive detection of both known and novel RNA viruses, the DECS-C method (dsRNA isolation, exhaustive cDNA amplification, and cloning-free next-generation sequencing) was employed as described by Yanagisawa *et al*. (2016). Briefly, dsRNA was extracted using a plant viral dsRNA enrichment kit (MBL, Aichi, Japan) according to the manufacturer's protocol. The TransPlex Whole Transcriptome Amplification (WTA) Kit (Sigma, St. Louis, MO, USA) was used to reverse-transcribe the extracted dsRNA into cDNA employing the supplied random primer and then to amplify the obtained cDNA following the manufacturer's protocol. PCR amplification was performed using ExTaq DNA polymerase (Takara Bio, Shiga, Japan) for a total of 20 cycles. The WTA products were purified using AMPure XP (Beckman Coulter, Brea, CA, USA). In total, 100 ng of WTA products was used to construct the sequence library via the TruSeq RNA Library Prep Kit (Illumina Inc., San Diego, CA, USA), and sequencing was performed on a MiSeq system (Illumina Inc., San Diego, CA, USA). The CLC Genomics Workbench ver. 11.0 (CLC Bio, Tokyo, Japan) was used for the analysis of sequence data. The mapping analysis was performed using the complete genomes of all viruses and viroids registered in the NCBI. The isolate obtained from a stunted seedling by the DECS-C method is hereafter referred to as the "PLV-OP isolate."

Plant growth conditions for virus inoculation

Plants of *Chenopodium quinoa* Willd. were grown in pots in a growth room at 25℃ under a 16-h photoperiod, while plants of *P. edulis* (passionfruit) were grown in a greenhouse. Three-week-old quinoa plants were used for inoculation with PLV-OP isolate and subsequent isolation. After inoculation, the plants were placed under fluorescent lights in a cool, air-conditioned laboratory for the observation of symptom development.

PLV inoculation and isolation

For the isolation of PLV, the PLV-OP iso-

late- originally obtained from a stunted passionfruit seedling and identified through DECS-C analysis- was used throughout. Quinoa plants were first inoculated with the sap of PLV-OP-infected passionfruit leaves using carborundum following the standard protocol. Then, single lesion isolation was performed through six repeated inoculations on quinoa plants. Finally, 10 g of leaves were collected for viral particle purification. The viral particles were partially purified as previously described in Atsumi *et al*. (2015). PLV-specific proteins were confirmed by SDS-PAGE using 5–20% e-PAGEL (ATTO Corp., Tokyo, Japan) and TaKaRa CBB Protein Safe Stain (Takara Bio, Shiga, Japan).

Testing PLV transmission by aphids

Cotton aphids (*Aphis gossypii* Glover) and green peach aphids (*Myzus persicae* Sulzer) raised cumulatively on healthy sweet pepper (*Capsicum annuum* L.) and eggplant (*Solanum melongena* L.) plants were fasted in a beaker for 2 h prior to allowing PLV acquisition via sucking. Specifically, using a brush, the fasted aphids were transferred to fresh upper leaves of passionfruit collected from plants infected with the PLV-OP isolate and were allowed to suck the sap for approximately 10 min. Then, a total of 10 or 20 aphids per plant were used for the inoculation of healthy passionfruit seedlings. The day after inoculation, Starkle® granules (1% granular dinotefuran, Hokko Chemical Co., Ltd., Tokyo, Japan) were applied at the base of each plant and, once the aphids were confirmed dead, the seedlings were moved into a glass room for the observation of the development of disease symptoms.

Testing PLV transmission via pruning shears

A passionfruit vine infected with the PLV-OP isolate was cut using pruning shears; then, the same shears were used to prune a healthy passionfruit seedling vine with approximately two to three true leaves. The pruned sapling was placed in a glasshouse and, after 1 mo, was subjected to both the examination of disease symptoms and testing for PLV-OP infection by RT-PCR. The experiment was conducted twice, in 2019 and 2020. In the 2019 trial, 18 replicates were performed, while 35 replicates were conducted in 2020, resulting in a total of 53 replicates over the two years.

RESULTS

Virus-like symptoms observed in passionfruit fields during the 2017 survey

In Okinawa Prefecture, abnormal virus-like symptoms had been observed in passionfruit during the winter/spring season prior to 2017 and had become a problem. Thus, this prompted a survey of virus diseases in passionfruit fields in this region in 2017. The four most common symptoms identified across Okinawa Island were (A) leaf curling (50 samples), (C) leaf surface cracking (26 samples), (D2) yellowing between leaf veins (22 samples) , and (B1) new shoot stunting (19 samples) (Table 1, Fig. 1). In contrast, the lowest incidence was observed for (F) fruit malformation (3 samples), followed by (B2) leaf stunting (5 samples) and (E) reduced vigor (6 samples). In total, 144 symptom observations were recorded from 104 passionfruit plants evaluated for visible abnormalities during this survey. This discrepancy reflects the fact that multiple symptoms were often observed on a single plant.

Distribution of passionfruit viral outbreaks in Okinawa Prefecture

In the 2017 survey, a total of 84 passionfruit fields (133 samples) in Okinawa Prefecture were investigated for the presence of virus-like infection in the leaves via RT-PCR tests. These fields were distributed as follows: 28 fields (39 samples) in the northern area of the main island, 3 fields (8 samples) in the central area, 36 fields (60 samples) in the southern area, 5 fields (10 samples) in Miyakojima Island, and 12 fields (16 samples) in the Yaeyama Islands. EAPDV was detected in 7 samples (2.4%, 5.3%) from 2 fields, EAPV-AO in 2 samples (1.2%, 1.5%)

from 1 field, and PLV in 107 samples (85.7%, 80.5%) from 72 fields (Table 3). Cases of infection with BBWV-2, CMV, and EAPV-IB were not confirmed. While symptoms in many infected plants were minor and had negligible impacts on cultivation, yet severe virus-like symptoms (e.g., fruit deformities) were observed in some plants in Uruma City and Ishigaki City. These cases involved overlapping infections: EAPDV and PLV in Uruma City whereas EAPDV, EAPV-AO and PLV in Ishigaki City. These observations demonstrated that passionfruit can be co-infected with three or more viruses. The survey also showed the frequent infection of passionfruit with PLV in Okinawa Prefecture and the presence, for the first time, of EAPV-AO in the area, highlighting the need to conduct periodic surveys of passionfruit viral outbreaks and to be vigilant for the emergence of new viral infections.

Distribution of PLV-infected passionfruit in Okinawa Prefecture in 2022

In 2022 survey, a total of 21 passionfruit fields (58 samples) in the southern part of the main island of Okinawa were investigated for PLV infection in the leaves via RT-PCR. The results showed that, since 2017, the incidence of PLV infections in Okinawa Prefecture markedly decreased from the previously high field rate of 85.7%. This was due to efforts to inform passionfruit farmers about the disease and to the introduction of virus-free seedlings obtained from Kagoshima Prefecture. Of the 15 fields planted with virus-free seedlings, three became infected with PLV which corresponded to an incidence of 20%. The survey of these 15 fields showed that 6 out of 39 (15.4%) samples were PLV-positive. Alternatively, of the six fields where virus-free seedlings were not used, five became infected with PLV, which corresponded to an incidence of 83.3%. The survey of these six fields showed that 15 out of 19 (78.9%) samples were PLV-positive (Table 4). For viruses other than PLV (i.e., BBWV-2, CMV, EAPV-AO, EAPV-IB, and EAPDV), no infections were identified in 21 fields (58 samples). This suggested that the spread of PLV in Okinawa Prefecture and the distribution of diseased seedlings were the main causes of the widespread occurrence of passionfruit virus outbreaks in the region. However, three of the 15 fields planted with virus-free seedlings were found to be re-infected with PLV despite the introduction of such seedlings. Based on interviews with growers after this survey, they confirmed that they had planted a mixture of self-propagated and virus-free seedlings. This suggested that either transmission by sap-sucking aphids or by juice remained on pruning shears may have occurred in the field.

Identification of PLV from a passionfruit nursery using the DECS-C method

Based on the 2017 survey result, PLV was considered as the primary pathogen, thus attempts were made to characterize and isolate it from diseased passionfruit. Comprehensive RNA virus detection techniques (i.e., DECS-C) were applied to passionfruit seedlings obtained from a seedling company that exhibited leaf morphological abnormalities (Fig. 2A). Deep sequencing yielded a total of 508,110 sequence reads. Mapping analysis was performed using reference sequences from approximately 7,000 viral genomes which resulted in a total of 35,369 reads being mapped to the reference genome of PLV (Fig. 3). For all the other viruses examined, the number of sequence reads mapped to their genomes was not sufficient to ensure reliable results. The consensus genomic sequence obtained from deep sequencing and mapping analysis showed 91.93% identity with the PLV isolate DSMZ PV-0222 (MT723990). Based on the result of sap inoculation on quinoa plants, the PLV isolate identified in this study caused necrotic spots on the inoculated leaves (Fig. 2B).

PLV inoculation and isolation

Single-lesion isolation was repeated 6 times before the systemically infected crude viral particles were subjected to purification. The crude viral particle fraction was separated by SDS-PAGE and stained with Coomassie

Table 3. Infected fields and rates of six viruses (in samples) already occurring in Japan in passionfruit fields in Okinawa, Japan, in 2017.

	BBWV-2[z]		CMV		EAPDV		EAPV-AO		EAPV-IB		PLV	
Survey location	Fields	Samples	Fields	Samples	Fields	Samples	Fields	Samples	Fields	Samples	Fields	Samples
The northern area of the main island	0/28[y]	0/39	0/28	0/39	0/28	0/39	0/28	0/39	0/28	0/39	26/28	33/39
Motobu Town	0/1	0/4	0/1	0/4	0/1	0/4	0/1	0/4	0/1	0/4	1/1	3/4
Onna Village	0/27	0/35	0/27	0/35	0/27	0/35	0/27	0/35	0/27	0/35	25/27	30/35
The central area of the main island	0/3	0/8	0/3	0/8	1/3	4/8	0/3	0/8	0/3	0/8	3/3	7/8
Nishihara Town	0/1	0/2	0/1	0/2	0/1	0/2	0/1	0/2	0/1	0/2	1/1	1/2
Uruma City	0/2	0/6	0/2	0/6	1/2	4/6	0/2	0/6	0/2	0/6	2/2	6/6
The southern area of the main island	0/36	0/60	0/36	0/60	0/36	0/60	0/36	0/60	0/36	0/60	35/36	56/60
Itoman City	0/33	0/54	0/33	0/54	0/33	0/54	0/33	0/54	0/33	0/54	32/33	50/54
Nanjo City	0/1	0/3	0/1	0/3	0/1	0/3	0/1	0/3	0/1	0/3	1/1	3/3
Yaese Town	0/2	0/3	0/2	0/3	0/2	0/3	0/2	0/3	0/2	0/3	2/2	3/3
Miyakojima Island	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10	3/5	5/10
Miyakojima City	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10	3/5	5/10
Yaeyama Islands	0/12	0/16	0/12	0/16	1/12	3/16	1/12	2/16	0/12	0/16	5/12	6/16
Ishigaki City	0/12	0/16	0/12	0/16	1/12	3/16	1/12	2/16	0/12	0/16	5/12	6/16
Sum	0/84	0/133	0/84	0/133	2/84	7/133	1/84	2/133	0/84	0/133	72/84	107/133
Incidence rate	0.0	0.0	0.0	0.0	2.4	5.3	1.2	1.5	0.0	0.0	85.7	80.5

[z] BBWV-2: Broad bean wilt virus 2; CMV: Cucumber mosaic virus; EAPDV: East Asian Passiflora distortion virus; EAPV-AO: East Asian Passiflora virus-AO; EAPV-IB: East Asian Passiflora virus-IB; and PLV: Passiflora latent virus.

[y] No. of virus infections/No. of reverse transcription-polymerase chain reaction (RT-PCR) tests.

Table 4. Number of infected passionfruit fields and rates of PLV infection in samples in Okinawa Prefecture, Japan, in 2022.

	No. of fields		No. of samples	
Seedling	Positive	Negative	Positive	Negative
Virus-free	3/15[z] (20.0)[y]	12/15 (80.0)	6/39 (15.4)	33/39 (84.6)
Not virus-free	5/6 (83.3)	1/6 (16.7)	15/19 (78.9)	4/19 (21.1)

[z] No. of PLV-infected fields (samples) by reverse transcription-polymerase chain reaction (RT-PCR)/No. of surveyed fields (samples).
[y] Parentheses indicate percentages.

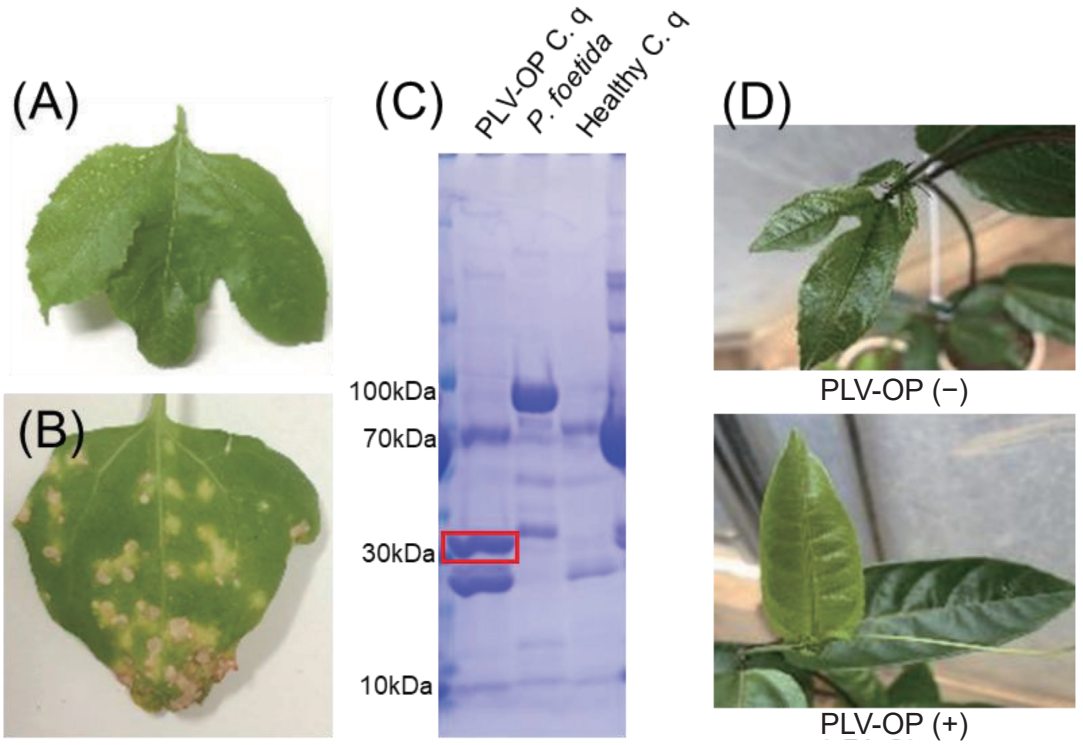

Fig. 2. PLV-OP isolated from a diseased passionfruit seedling. (A) Virus-like symptoms observed on the leaf of a diseased passionfruit seedling obtained from a commercial seedling supplier in Okinawa. (B) Necrotic spots on a leaf inoculated with PLV-OP. (C) Protein bands obtained from specific fractions during the purification of viral particles extracted from infected passionfruit plants. (D) Healthy appearance of passionfruit plants inoculated with PLV-OP (+) compared to non-inoculated plants PLV-OP (−).

Brilliant Blue (CBB) that revealed a band of approximately 30 kDa. This band was estimated to represent the coat protein of PLV (Fig. 2C). The isolated virus was subsequently confirmed by RT-PCR and designated as the Okinawa passionfruit isolate of PLV (PLV-OP). Next, mechanical inoculation tests were conducted to evaluate its pathogenicity. The virus was inoculated onto the mature leaves of passionfruit plants using a crude extract containing PLV. One week after inoculation, PLV was detected in the newly emerged, non-inoculated leaves by RT-PCR. However, no visible symptoms developed and the infected plants grew similarly to healthy controls (Fig. 2D). For grafting experiments, the virus-free seedlings were grafted with scions obtained from PLV-infected passionfruit plants with abnormal symptoms,

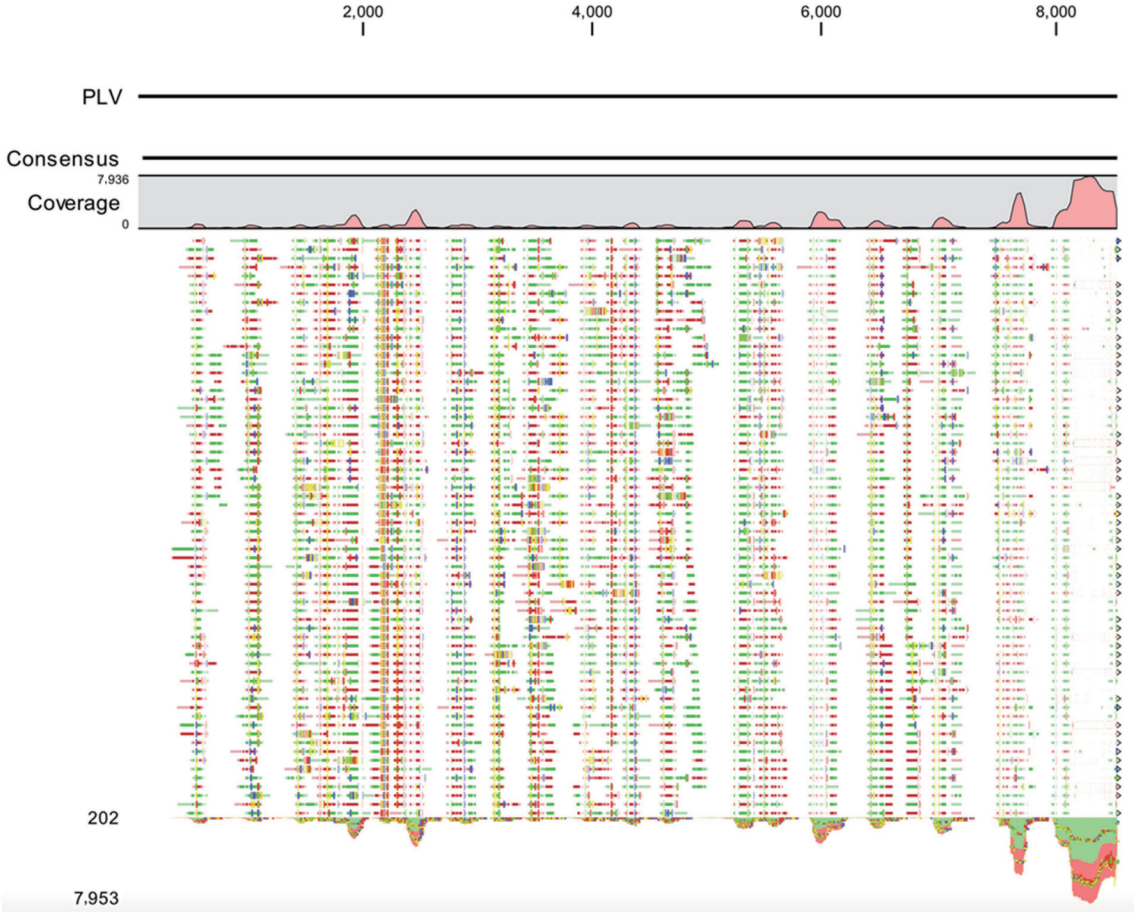

Fig. 3. Whole Passiflora latent virus (PLV) genome covered by sequence reads derived from dsRNA extracted from diseased passionfruit. The numbers indicate the depth of 35,369 mapped-reads analyzed using the CLC Genomics Workbench.

results showed that no reproduction of the specific symptoms previously observed in passionfruit during the winter/spring season in Okinawa Prefecture. Sap extracted from symptomatic tissues was used to inoculate healthy passionfruit seedlings yielded no similar symptoms as that observed in fields.

Testing PLV transmission by aphids

Propagation tests were conducted to investigate how PLV spreads in passionfruit fields. The ability of *A. gossypii* and *M. persicae* to transmit PLV to passionfruit plants was assessed. For *A. gossypii*, 10 or 20 aphids were allowed to feed on passionfruit plants infected with the PLV-OP isolate and subsequently transferred to virus-free passionfruit plants, no symptoms were observed in the recipient plants. However, RT-PCR detected the presence of PLV in 22.2% of the plants inoculated with 10 aphids and 16.7% of those inoculated with 20 aphids. In contrast, when *M. persicae* were used in the same manner, neither symptoms nor PLV infection were detected in the recipient plants (Table 5).

Testing PLV transmission via pruning shears

Tests were conducted to assess PLV-OP transmission in passionfruit plants via pruning

Table 5. Transmission of Passiflora latent virus (PLV) by aphids.

Aphid	Feeding period[z]	No. of insects[y]	Symptomatic plant[x]	Positive reaction[w]
Aphis gossypii	10 min	10	0/9 (0.0)[v]	2/9 (22.2)
A. gossypii	10 min	20	0/6 (0.0)	1/6 (16.7)
Myzus persicae	10 min	15	0/6 (0.0)	0/6 (0.0)

[z] After being fasted for 2 h, the aphids were allowed to feed on infected leaves for different periods and then transferred to the test plants, on which they continued to feed overnight.
[y] No. of insects used for inoculation per plant.
[x] No. of symptomatic plants/No. of inoculated plants.
[w] No. of PLV-positive plants by reverse transcription-polymerase chain reaction (RT-PCR)/No. of inoculated plants.
[v] Parentheses indicate percentages.

shears. In the 2019 and 2020 surveys, no symptoms were observed, although RT-PCR results showed infection rates of 94.4% and 8.6%, respectively. Throughout the 2-year survey, the average rate of PLV infection by pruning shears was confirmed at 37.7%, although no viral symptoms were identified in any of the samples (Table 6).

Table 6. Transmission of Passiflora latent virus (PLV) by contaminated pruning shears.

Experiment year	Symptomatic plant[z]	Positive reaction[y]
2019	0/18 (0.0)[x]	17/18 (94.4)
2020	0/35 (0.0)	3/35 (8.6)
Average	0/53 (0.0)	20/53 (37.7)

[z] No. of symptomatic plants/No. of inoculated plants.
[y] No. of PLV-positive plants by reverse transcription-polymerase chain reaction (RT-PCR)/No. of inoculated plants.
[x] Parentheses indicate percentages.

DISCUSSION

Based on the 2017 field survey, PLV was detected in 107 out of 133 samples (80.5%), making it the most prevalent virus in Okinawa Prefecture. However, symptom observation did not show a consistent pattern associated with PLV infection alone. As indicated in Fig. 1 and Table 1, PLV-positive plants exhibited various symptoms including leaf curling, interveinal chlorosis, new shoot stunting, and fruit malformation. These symptoms were generally diverse, mild to severe and non-specific, suggesting that PLV infection alone may not fully account for the abnormalities observed in the field.

Furthermore, mechanical inoculation using purified PLV-OP isolate (Fig. 2D) did not reproduce visible symptoms in passionfruit plants, even though systemic infection was confirmed by RT-PCR. This reinforces the hypothesis that PLV may behave as a latent or weakly pathogenic virus, with visible symptom expression dependent on environmental conditions, host factors, or viral co-infection.

Supporting this interpretation, Table 3 shows that severe symptoms such as fruit deformation and shoot distortion were observed in Uruma City and Ishigaki City, where PLV co-occurred with EAPDV and/or EAPV-AO. These cases suggest that co-infection may intensify symptom severity, a phenomenon commonly reported among Carlaviruses. Therefore, effective disease diagnosis and management should consider both viral prevalence and the possibility of co-infection in affected fields.

The survey results indicated a possible correlation between the large-scale growth abnormalities in passionfruit cultivated in Okinawa Prefecture and PLV infection. However, in this study, the back-inoculation test using the PLV-OP isolate did not reproduce the pathological symptoms. As a result, Koch's postulates for confirming the pathogen's role were not fulfilled. Viruses belonging to the genus *Carlavirus*, such as PLV, often require co-infection with other viruses to manifest symptoms. For example, Carnation latent virus (CLV) in Japanese carnations typically remains asymptomatic but causes symptoms when co-infected with Carnation vein mottle virus (Morita &

Horie 1998). Similarly, Lily symptomless virus in lilies remains latent but leads to guttate lesions when co-infected with CMV (Nishi *et al*. 2008). Therefore, it is possible that PLV alone does not cause severe damage in passionfruit but may do so when combined with other viruses. In this study, no other viruses were detected in the PLV-infected passionfruit plants using the DECS-C method. Previous studies on PLV's virulence in passionfruit (Bao *et al*. 2023; Choi & Ju 2023) have shown varying results some with strong virulence while others with minimal or no impact. These discrepancies suggest that different PLV strains might contribute to the varying symptoms observed in passionfruit. Moreover, the pronounced symptoms during winter and spring hint at the significant influence of environmental factors on disease development.

In another region of Japan, Gifu Prefecture, a survey on viral diseases on passionfruit was conducted similar to the study in Okinawa Prefecture. The survey in Gifu Prefecture revealed that 72.1% of passionfruit plants were infected with PLV (Muramoto & Suzuki 2018). Additionally, the survey reported a high incidence of CMV outbreaks which were not detected in Okinawa Prefecture. This difference in CMV incidence between the two prefectures may be attributed to differences in cultivation methods. In Okinawa Prefecture, passionfruit is cultivated indoors due to high rainfall which decreases susceptibility to pests and diseases. In contrast, in Gifu Prefecture, passionfruit is grown in open fields where aphid infestations are more likely to occur (Kumamoto *et al*. 2017). In Gifu Prefecture, CMV infection rates are reduced when aphids are controlled. Furthermore, because CMV causes clear leaf atrophy symptoms, it is unlikely that infected seedlings (and thus the virus) are introduced into fields (Muramoto & Suzuki 2020). Therefore, it is inferred that CMV contamination occurs after seedlings are planted, primarily through aphid transmission. High CMV infection rates have been observed in Gifu Prefecture, where aphids easily infest passionfruit in open fields. Whereas, no cases of CMV were detected in Okinawa Prefecture where passionfruit is cultivated indoors. Based on these findings, the present study also examined the possibility of PLV transmission via two aphid species as well as sap-contaminated pruning shears. The results indicated that *A. gossypii* transmitted PLV in a non-persistent manner. Non-persistent transmission means that the virus is spread by aphids during short feeding sessions, without the virus being retained for long periods. Viruses belonging to the genus *Carlavirus*, including PLV, have been reported to be transmitted in this manner. Specifically, Omatsu *et al*. (2004) confirmed non-persistent transmission of EAPV by the sowthistle aphid (*Nogeshi-fukure-aburamushi*; *Hyperomyzus carduellinus*), cotton aphid, and peach aphid.

Among the studies conducted on the transmission of passion fruit viruses by aphids, Parry *et al*. (2004) and Yonaha *et al*. (1979) reported infections with Passiflora virus Y (PaVY) via cotton aphids and the non-persistent transmission of CMV by peach aphids, respectively. These reports indicate that aphid control is important for mitigating the spread of passionfruit virus diseases including PLV. However, in recent years, PLV infections have been confirmed in many passion fruit farms in Okinawa Prefecture, although these were all within greenhouse environments where aphid infestations have been rarely reported. Therefore, it is unlikely that aphids are an important route of PLV transmission in this prefecture. However, this study showed that sap-contaminated pruning shears represented an effective means of PLV transmission, especially in passionfruit which requires frequent pruning. This suggested that once the virus enters a field, it can spread across it through cultivation management practices.

In addition, measures against passionfruit virus disease have been thoroughly promoted by conducting RT-PCR tests on samples obtained from seedling suppliers in Okinawa Prefecture. These efforts, which have been ongoing since 2019, aim to identify and prevent

the spread of infected seedlings. This approach complements the findings of the present study, as it highlights the importance of ensuring the distribution of virus-free seedlings to mitigate disease outbreaks.

In summary, the present study revealed widespread viral infections in passionfruit fields in Okinawa Prefecture, with PLV identified as the major virus transmitted via aphids and contaminated pruning shears. The introduction of virus-free seedlings significantly reduced the incidence of PLV during the growing season, indicating that the distribution of infected seedlings was the primary source of infections. However, when virus-free seedling fields are adjacent to fields with non-virus-free seedlings, or in fields where virus-free and non-virus-free seedlings are grown together, controlling aphids and disinfecting pruning shears become essential. Comprehensive and integrated control strategies will be crucial for managing passionfruit virus diseases effectively in the future.

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日本沖繩百香果病毒調查：百香果潛隱病毒的流行、傳播及利用去病毒苗達有效防治

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摘要

Ajitomi, A., A. Saito, S. Kawano, and K. T. Sekine. 2025。日本沖繩百香果病毒調查：百香果潛隱病毒的流行、傳播及利用去病毒苗達有效防治。台灣農業研究 74(3):275–289。

在日本沖繩縣，冬春季節種植的百香果出現新葉枯萎、植株活力下降等徵狀的栽培問題。2017 年，調查 84 個田區而確認了疑似病毒病的徵狀，並以 reverse transcription-polymerase chain reaction (RT-PCR) 進一步調查日本已報告的 6 種特定病毒的疫情。結果顯示，最常見的 3 種病徵是成年葉片捲曲、葉面開裂及葉脈間黃化。在檢測的 6 種病毒中，東亞百香果皺縮病毒 (East Asian Passiflora distortion virus; EAPDV)、東亞百香果病毒 -AO (East Asian Passiflora virus-AO; EAPV-AO) 與百香果潛隱病毒 (Passiflora latent virus; PLV) 的發生率分別為 2.4%、1.2% 及 85.7%，而蠶豆萎凋病毒 -2 (Broad bean wilt virus-2; BBWV-2)、胡瓜嵌紋病毒 (Cucumber mosaic virus; CMV) 均未檢測到。感染 EAPDV 與 EAPV-AO 的植物表現出葉片皺縮與果實畸形的典型徵狀，而感染 PLV 的植物則表現出其他非特異性徵狀。根據調查結果，PLV 被確定為主要病原。此病毒是從受感染的百香果中所分離而得，可導致奎藜出現病徵，但不會在百香果引起病徵。PLV 可透過昆蟲媒介 (例如棉蚜) 以及修枝剪所殘留的汁液傳播。此外，由於在從種苗供應商的百香果幼苗中檢測到了 PLV，因此從 2019 年開始，沖繩縣採取了供應已確認不含 PLV 的幼苗措施。此後，PLV 的發生率急劇下降，2022 年所進行的第二次田間調查顯示，受 PLV 感染的田地數量明顯減少。沖繩百香果的異常徵狀與 PLV 感染呈現較強的正相關性。

關鍵詞：蚜蟲、修枝剪、RT-PCR、DECS-C、*Carlavirus* 病毒屬。

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