Studies on molecular classification and propagation of a new species of potyvirus infecting Passifloraceae plants

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CONTENT

I. SUMMARY

Passionfruit (*Passiflora edulis* Sims) is one of more than 500 species of the *Passiflora* genus, a perennial evergreen tree from South America that climbs naturally. Like other tropical crop, several diseases have led to significant losses in passionfruit yield. Prior to this study, a causal woodiness disease by a new species of potyvirus, *East Asian Passiflora virus* (EAPV), was detected in passionfruit plants in Japan but could be mostly reduced until the end of 2010. During 2013 to 2016, a typical virus causing foliar mosaic, leaf curl and fruit malformation was detected in passionfruit from three prefectures in Japan. RT-PCR amplification of the samples in the CP coding region using universal primer of potyvirus shown that they are belong to the potyvirus genus. The present work was aimed primary for investigating the biological properties, complete sequences of the isolates of novel potyvirus, and its propagation coinfected with EAPV in *Passiflora foetida*, a wild species of passionfruit.

Viruses were collected from Akita City, Akita Prefecture in 2013, Nago City, Okinawa Prefecture in 2014, and Satsuma Town and Yoron Island, Kagoshima Prefecture in 2015. The isolates were designated PV-AK (Akita), PV-OK (Okinawa), PV-YO (Yoron), and PV-SA (Satsuma). Electron microscopy analysis revealed that the isolates contained a flexuous, filamentous particles with the average length was 810 nm. In the host-range test, four of 13 cultivars of French bean were systemically infected with the PV isolates and two with EAPV-AO (Amami Oshima strain). Cowpea cv. Kurodane sanjaku was infected systemically with PV isolates and induced mosaic symptoms on non-inoculated leaves but was immune to EAPV-AO.

The complete genomic sequence of the PV isolates was constructed using PCR with specific and degenerate potyvirus primers consisted of 9973 nucleotides (nt) excluding the poly(A) tail, and encoded 3217 amino acids of polyprotein, flanked by 129–130 nt of the 5'-noncoding region and 193 nt of the 3'-noncoding regions. The sequences were submitted to GenBank under accession numbers PV-AK (LC379162), PV-OK (LC373083), PV-YO (LC377302), and PV-SA (LC375413). Whole-genome nucleotide and amino acid sequences of PV isolates indicated that they belong to identical species. The potyvirus with the highest whole genome nucleotide identity to the PV isolates is watermelon mosaic virus, which shares 68.1% identity, versus 65.3% for EAPV-AO. Since ICTV-naming regulations, these PV isolates belong to a new species in the *Potyvirus* genus but are distantly related to EAPV. East Asian Passiflora distortion virus (EAPDV) was proposed as the new species name.

In this study, the ability of EAPDV and EAPV to interact synergistically in mixed infection in *P. foetida*, a wild species of *Passifloraceae* which is easily infected with both viruses by mechanical inoculation, has been examined. Dual inoculation of EAPDV and EAPV results in different symptoms and viral accumulation on *P. foetida*. Mixed inoculation and EAPV followed by EAPDV inoculation were synergistic resulting in enhancement symptoms severity on the leaves but no change in the level of accumulation of CP of EAPV. In contrast, EAPDV followed by EAPV inoculation delayed the symptom severity and had a significant accumulation of CP of EAPDV. In case of pathogenesis related (PR) protein which may associate with symptomatology and plant cell death program of the infected plant, mixed and two-step inoculation of EAPDV and EAPV elevated lipoxygenase-2 (*LOX2*) gene expression whereas mixed infection,

but not two-step infection, elevated cathepsin B-like cysteine protease expression. In short, we found that the existence of EAPV induced EAPDV propagation and that *P. foetida* is a potential reservoir for EAPDV and EAPV.

II. INTRODUCTION

Passionfruit (*Passiflora edulis* Sims) is a species of *Passifloraceae* that is native from southern Brazil to northern Argentina. This perennial climbing vine plant is grown widely in subtropics to tropical regions, and it is cultivated industrially in Australia, Hawaii, Malaysia, Sri Lanka, Taiwan and Vietnam (Cerqueira-Silva et al. 2015). This species is classified into the purple type (*Passiflora edulis*) and yellow type (*P. edulis* f. *flavicarpa*) by its fruit color. The purple type of passionfruit is strongly sweet and consumed as an expensive raw fruit. The yellow type is rich in juice and is grown mainly for processing. This exotic fruit was introduced to Ibusuki City in Japan's Kagoshima Prefecture in the 1920s. The Nansei islands (a chain of islands extending southwest of Kyushu Island, Japan) became the center of passionfruit cultivation in the late 1950s. After the 1980s, large-scale commercial cultivation became common on Amami Oshima Island in Kagoshima Prefecture and the main island of Okinawa Prefecture (Iwai et al. 1996). At present, the hybrid passionfruit species Ruby star and Summer queen (*P. edulis* x *P. edulis* f. *flavicarpa*) are grown mainly on Amami Oshima Island (Iwai et al. 2006), and in recent years, this cultivation has also been developed in Tokyo (Ogasawara Islands), Chiba and Gifu Prefectures due to the application of temperature-controlled facilities. From now on, it will be referred to hybrids as "passionfruit" unless otherwise noted.

Several viruses have been reported to pose a significant obstacle to the yield and quality of passionfruit worldwide. In Japan, there are four species of virus naturally infecting passionfruit. These are cucumber mosaic virus (Yonaha et al. 1979), broad bean wilt virus (Yonaha et al. 1993), East Asian Passiflora virus (EAPV) (Iwai et al. 2006),

and Passiflora latent virus (Watanabe et al. 1997). Of these four viruses, EAPV-AO (Amami Oshima strain), a species in *Potyvirus*, is a causal pathogen of passionfruit woodiness disease (PWD). Another EAPV strain, EAPV-IB (Ibusuki strain), was also found in Kagoshima Prefecture (Iwai et al. 2006), but the symptoms caused by IB were not severe and its distribution was very narrow.

In Japan (Southern Kyushu in particular), the production of passionfruit had been prevented by EAPV-AO since 1986 but the disease was mostly reduced by around 2010 due to large-scale replanting with healthy plants (Iwai 2017). However, since 2013 to 2015, a new type of *Potyvirus* causing identical foliar mosaic, leaf curl, and fruit malformation was found infecting local hybrid varieties of passionfruit (*P. edulis* x *P. edulis* f. *flavicarpa*) consecutively in Akita Prefecture, Okinawa Prefecture, and two administrative areas in Kagoshima Prefecture.

Initially a type of *Potyvirus* in passionfruit plants was first detected in Akita in 2013 but this disease thought to have originated in southern Japan. Moreover during 2014 to 2015, several similar disease symptoms from passionfruit were received from passionfruit growing in Yoron and Satsuma district, Kagoshima prefecture and Nago city, Okinawa prefecture.

After collection of several symptomatic passionfruit from Nago city, Agriculture research center of Okinawa prefecture removed all infected plants and distributed healthy plants to commercial field in the area. However, since then, on some islands of Okinawa prefecture, the new *Potyvirus* is still prevalent. In addition, passionfruit carrying EAPV have also been found in Okinawa prefecture. Mixed infection is a natural phenomenon that happens in nature. Propagation of a plant virus in presence of other viruses generate

a specific interaction in plant. The interaction will influence the development and survival ability of the virus in the plant. The type of interactions between different viruses are generally synergistic or antagonistic. Synergistic is when interactions cause symptoms in plants to become more severe whereas antagonists are the opposite.

P. foetida was used to investigate the interaction of both viruses due to the difficulties of passionfruit to be mechanically inoculated. This species which like passionfruit is a wild species of *Passifloraceae*, originally from South America and is called by various names such as bush passionfruit, running pop, and wild maracuja, and has already been naturalized in a wide area of the western Pacific from the Okinawa islands of Japan to Indonesia. This wild passionfruit is easily infected with EAPV by mechanical inoculation. Subsequently, *P. foetida* has been reported to be naturally infected with many plant viruses (Davis et al. 2002; Leggat and Teakle 1975; Parry et al. 2004). Leggat and Teakle (1975) explained that this plant appears to be a reservoir of passionfruit woodiness virus (PWV) in Australia. So far, no natural potyvirus infection has been found in *P. foetida* in Japan, but Yonaha et al. (1979) found infection of cucumber mosaic virus naturally in this plant. Thus, making this plant also to be a potential species as a virus-reservoir plant in Japan.

In light of the above background, my study was aimed at to investigate the biological and molecular characterization of four *Potyvirus* isolates from passionfruit leaves showing mosaic and leaf curl symptoms, and to study the propagation of the new *Potyvirus* isolate and EAPV in *P. foetida* through measuring the accumulation of both viruses by real-time PCR, and compared the results with those of single infections followed by discussion of plant roles related to the spread of viruses in the field.

III. LITERATURE REVIEW

3.1. Viruses

The virus is a small and simplest infectious agent that efficiently replicates inside living cell. Hull (2002) defined clearly that a virus is “a set of one or more nucleic acid that is able to organize its own replication only within suitable host cells”. As the simplest feature, the virus plague yields countless number of death (Institute of Medicine 2009). Viruses can cause crop losses worth up to 30 billion dollars each year to crops (Sastry and Zitter 2014). Anderson et al. (2004) also calculated that viruses cause just under half (47%) of such diseases.

The number of viruses in nature increases very rapidly. Almost every day, a novel virus is registered in Blast search. Environmental changes, an increase in the human population, at that time there was an exchange of plant material and products are several factors of the rapid spread of previously localized viruses. Metagenomic type survey indicated that true diversity of virus is much larger than previous estimates (Labonte and Suttle 2013). One type of virus is RNA viruses. This virus type is a small genome size virus which an average 3 to 10 kb, it generally tolerates higher levels of mutagenesis (Domingo and Holland 1997) as compared to DNA viruses. The type mutates rapidly because due to their error-prone replication as result of no proofreading activity in RNA-dependent RNA polymerase (Drake and Holland 1999). The mutation ability is beneficial for viruses to effort in adapting to new hosts.

Generally, the number of viruses in one genus is quite large, because of that classification is a challenge. Accurate diagnosis and rapid detection are the major factor needed for obtaining successful management and control effectively. One of the initial

criteria for distinguishing viruses is host range assay and symptom expression. Although different host may show distinctive symptom by infection of some virus, many plant viruses can infect a wide range of plant hosts (Khan et al. 1991; Moran et al. 2002) and a single host species may be susceptible to many different viruses (Brunt et al. 1996), the distinguishing criteria according to the host range is still reliable in grouping a species.

At early technological developments, serological techniques (Ainsworth 1981), virus purification methods and invention of electron microscope which enabled viruses to be visualized substantially increased the ability to detect and identify a virus. A major method made years after that is use of enzyme-linked immunosorbent assay (ELISA) (Thresh et al. 1977). Then developing of monoclonal antibody technology and the application followed by developing a different number of variants of ELISA, invented recent, can identify various viruses of many crops efficiently (Aboul-Ata et al. 2011).

In the late of 1980s, since the introduction of northern blot in 1977, the development of advanced molecular techniques based on reverse transcription polymerase chain reaction (RT-PCR) then followed by sequence techniques invention were applied for detection of many viruses. The use of the RT-PCR is five times sensitive as ELISA. In 1990s

, the RT-PCR has been used to study analytically of viral genome, quasi-species-like structures in population, and relationships among viral strains and species. Analysis based on capsid protein or polyprotein sequences and the phylogenetic analysis are the most current classification and more balanced approach and gives the whole view of how genome relatedness (Stuart et al. 2004).

3.2. Viruses infecting *Passiflora edulis* naturally

The earliest identified virus that affected passionfruit was PWV in Queensland (Australia) (Taylor and Kimble 1964). This virus is belonging to genus of *Potyvirus* and as one major causal agent in passionfruit disease. In Japan, around 1986, a similar PWD was observed in Setouchi, southern part of Amami Oshima island, Kagoshima prefecture. Initially, my laboratory identified the cause of this PWD as PWV-AO (Iwai et al.1996). However, after identification using genome analysis, the virus has low similarity in the amino acid sequences of the CP of this isolate with PWV and it was reported as a new *Potyvirus* species causing PWD and named East Asian Passiflora virus (EAPV), which consist of two strains causing different symptom appearance, strain AO and IB (Iwai et al. 2006). Even in Taiwan before 1990, the virus responsible for PWD was thought to be PWV (Chang 1992), but recent reports state that the PWD in Taiwan is also caused by EAPV (Chong et al. 2018), which causing more wider yield loss (Tsai et al. 2019). In addition, EAPV-AO and EAPV-IB have been found in Malaysia and Korea, respectively (Abdullah et al. 2009; Hong et al. 2015). More recently, EAPV was appeared in Vietnam even two strains of EAPV-AO and IB were detected (Hung et al. 2019). In foreign countries, other potyviruses causing PWD have been reported, such as *Cowpea aphid-borne mosaic virus* (CABMV) in Brazil and South Africa (Nascimento et al. 2006), and Ugandan passiflora virus in Uganda (Ochwo-Saemakula et al. 2012).

3.3. The potyvirus

Potyvirus is the major causal virus group causing significant losses of crops in worldwide (Gibbs and Ohsima 2010). First diverged of potyvirus was 7250 years ago originated from a virus of monocotyledonous plants in Southwest Eurasia or North Africa

(Gibbs et al. 2008). The emergence is triggered by the dawn of agriculture (Gibbs et al. 2008). One *Potyvirus* may be spread by 200 aphids and vice versa, an aphid can transmit many *Potyvirus* species. The aphids transmitted the potyvirus mostly by non-persistent manner, making them difficult to control (Gibbs et al. 2003; Poutaraud et al. 2004).

Potyvirus is the largest of eight genus of family *Potyviridae* with more than 160 species, which most of them has narrow host range (Hajimorad et al. 2018; Shan et al. 2018). The genus is declared to be mostly belonging to bean common mosaic lineage (Gibbs and Ohshima 2010; Gibbs et al. 2008). Their virions are flexuous, nonenveloped, rod-shaped particles, 680-900 nm long and 11-15 nm wide (Urcuqui-Inchima, et al. 2001). The genome is a single stranded positive sense (+)ssRNA. At first replication stage, RNA polymerase translated genomic (+)ssRNA into (-)RNA, and the (+) RNA copied from (-)RNA is the offspring. A (+)ssRNA is translated into about 350 kDa in size of polyprotein which is flanked by the 5'-non coding region (NCR) and a poly-A tail at the 3'-NCR. The polyprotein is subsequently cleaved by three virus-encoded proteinase into 10 proteins: P1 (P1 protease), HC-Pro (helper component-proteinase to mediate insect transmission, viral replication, suppression of Post Transcriptional Gene Silencing and cell to cell movement), P3 (to have a role in viral replication, movement, virulence and avirulence activities), 6K1 (unclear function), CI (cylindrical cytoplasmic inclusion and cell to cell movement), 6K2 (unclear function), VPg (genome-binding protein), Pro (NIa protease), NIb (RNA polymerase) and CP (coating the virion) (Cui et al. 2017; Shan et al. 2018; Govier and Kassanis 1974; Plisson et al. 2003; Eggenberger et al. 2008; Deng et al. 2015; Martinez et al. 2016; Hong and Hunt 1996; Gough and Shukla 1981). One of *Potyvirus* characteristic features is that virus–encoded cytoplasmic cylindrical inclusion

(CI) bodies forrmed in the cytoplasm (Van Regermortel 2000).

The potyvirus individually has narrowed host range, but in a group, they have a wide range of crops (Hollings and Brunt 1981). The hosts of potyvirus listed by Brunt et al. (1996) included 503 plant species belonging to 59 plant families, most of which are economically important plants. In addition, new hosts are continuously reported (Arli-Sokmen et al. 2005; Morris et al. 2006; Parry and Persley 2005). Potyviruses caused a wide range of different symptoms in infected host plants, including mosaic, stripe, mottling, vein clearing, vein banding, ringspots, necrotic or chlorotic lesions, flower breaking, necrosis, stunting, wilting, of which stunting and yield losses are most common with a combination of several symptoms is usually found in both natural and experimental hosts (Shukla et al. 1994).

3.4. Propagation of plant viruses

Propagation of multiple virus in a plant are common in nature. Multiple interaction during propagation of each causative agent caused many important virus diseases in plants. These infections generate a variety of intra-host virus-virus interaction leading a novel feature and change of genetic structure of viral population. Consequently, study of interactions is very important to understand the pathogenesis of a virus and specially to obtain efficient control.

3.4.1. Synergistic interaction

Synergistic is the most common interaction. This interaction increases the titers of both or at least one virus in the host plant (Hull 2002) and intensified symptom to be more severe than that induced by single virus infection (Senanayake and Mandal 2014).

Synergies can occur between unrelated viruses and viroid, viruses and RNA or satellites (Sanger et al. 1994; Scholthof 1999). Synergistic interaction of potyvirus and unrelated virus mostly induces an increase the titer of unrelated virus (Hameed et al. 2014; Wang et al. 2009b). For example, titer of potato virus X (PVX) had up to 10 times of PVY titer, while titer of PVY remained the same either conditions (García-Marcos et al. 2009).

Other certain of synergistic relation is both viruses can gain benefits. For example, co-infection of corn maize chlorotic mottle virus (CMCV) and wheat streak mosaic virus (WSMV) show an increase their titers up to 23 folds in *N. tabacum* (Gonzalez-Jara et al. 2004). Pathway in potyvirus are usually mediated by the expression of helper component-proteinase. When infection occur, PTGS degrade virus RNA by using small interfering RNA (siRNA). HC-Pro plays to silence the suppressor of host PTGS. In simultaneous infection, TuMV co-infects with lettuce infectious yellow virus (LIYV) result in high replication efficiency and titer of LIYV (Wang et al. 2009b). It was confirmed by transgenic plant of P1/HC-Pro inoculated with LIYV that TuMV has silencing suppressor roles and increase titer of LIYV. However, there is unclear the consensus of mixed infections between potyviruses or with homologues of potyvirus group.

3.4.2. Antagonistic interaction

Antagonistic interaction is characterized by one of the viruses gain benefit, and its presence and activity reduce the suitability of the second virus. During the interactions, one virus lowering the fitness and accumulation of the second virus (Syller 2012). There is only two case of antagonistic interaction found, where CP accumulation of one of the viruses is decreased. Antagonistic interactions also called cross-protection or

superinfection can be used as vaccines in plants. Mild type of virus can induce plant resistance to second viruses that infect latter (Syller 2012). The more relationship between viruses, the greater chance of cross-protection (Roossinck 2005). For example, beet soilborne mosaic virus (BSBMV) and beet necrotic yellow vein virus (BNYVV), both from the *Benyvirus* genus, BSBMV protect plant from invasion of BNYVV up to 100% at interval 10 days (Mahmood and Rush 1999). As well cross-protection among different isolates of WSMV. Mild type of isolates of WSMV-Kmi co-infect with WSMV-A or WSMV-Ks reducing symptom severity of the plant (Slykhuis and Bell 1966). Syller (2012) explained that by mutual exclusion, refer as spatial separation, antagonistic strains or species avoid coexistence in the same cell. For example, labeled apple latent spherical virus strain (ALSV-CFP) was always separated with ALSV-YFP in the leaves.

3.4.3. Expression of pathogenesis-related (PR) encoding gene

Plants during plant virus propagation in their tissue have developed complex signaling and defense mechanisms to defend themselves against pathogen invading. Beside modifications of morphological and physiological feature, multiple signaling pathways will be stimulated leading to the activation of defense mechanisms. Some of them are the induction of PR proteins, hypersensitive reaction and cell death programs (van Loon et al. 1994. Park et al. 2002).

Systemic necrosis is one of the characteristics of plant hypersensitive responses because of delays in biochemistry and physiology. Several PR-protein encoding gene through various pathway is produced in response to, necrotizing particularly, infection by virus (van Loon and Gerritsen, 1989; Park et al., 2002). Lipoxygenase (*LOX*), an enzyme involves in the jasmonic acid pathway is a family of iron-containing dioxygenases which

catalysis the hydro oxidation of lipids, containing a cis, cis-1,4-pentadiene structure. *LOX* gene can be classified as 9- and 13-lipoxygenases, according to the position of oxygen which incorporated into linoleic acid and linolenic acid, the most important substrates for *LOX* catalysis in plants (Feussner and Wasternack, 2002).

13-LOX in linolenic acids results in a Methyl Jasmonate (Me-Ja) and Isoleusine Jasmonate (Ile-Ja) and serves the key rules of transduction pathway signals that regulate gene expression related to defense and development. *9-LOX* participates in several aspects of plant development and defense through the JA independent signal pathway. Some examples of oxylipin derived from activity *9-* and *13-LOX* are to initiate plant cell death (PCD) and hypersensitive responses (Rustérucci et al. 1999). Systemic necrosis arising from infection of *N. benthamiana* infection by PVX and PVY related to the presence of oxylipin biosynthetic pathways (García-Marcos et al. 2013).

Cathepsin B is a member of cysteine proteases found in lysosomes in animal cells (Mort and Buttle, 1997). The protein was found to mediate PCD and be associated with many diseases. Not many research concerns on plants cathepsin B but this gene is also found to be an important component of PCD. Transcript level of cathepsin B transcript was found in barley aleurone layers in response to incubation with Gibberellic Acid (GA3). During seed development, PCD was found to occur in aleurone cells (Young and Gallie 2000; Fath et al. 2000). Beside that Abscisic acid (ABA) was also found antagonistically regulate PCD in aleurone cells (Lovegrove and Hooley 2000). These combinations shown that cathepsin B involves regulating PCD.

Other more reports provide the evidence for cathepsin B regulation of PCD in plants. Cathepsin B paralogues was found in Arabidopsis having a function in pathogen

defense (McLellan et al. 2009). One of the paralogues cathepsin B show significant in HR-induced PCD of Arabidopsis. Other evidence confirmed that after inoculation of *Pseudomonas syringae* DC3000 to Arabidopsis, this gene shows a different expression level. Gillroy et al. (2007) measured cell death in *N. benthamiana* inoculated with *Erwinia amylovora* (Eam). *P. syringae* pv. tomato (Pst) was suppressed by Virus Induced Gene Silencing (VIGS) of cathepsin B. The cathepsin B transcript level and activity was induced in an apple infected by Eam and cathepsin B inhibitors (CA074me) suppressed Eam-induced HR PCD. The relevance of protease activity was also found in plant virus interaction few years ago. It was explained by Hanssen et al. (2011) that cathepsin B in tomato was induced 146-fold by the aggressive isolate of pepino mosaic virus (PMV) and induced only 100-fold by the mild isolate even the viral titers were similar.

IV. MATERIAL AND METHODS

4.1. Sources of materials

The plant material using for host range assay belonging to ten species of seven plant families were: *Capsicum annuum* cv. California wonder, *Chenopodium amaranticolor, C. quinoa, Datura stramonium, N. benthamiana, N. glutinosa, N. tabacum* cv. Bright Yellow, *P. edulis, P. edulis* x *P. edulis* f. *flavicarpa, P. foetida, Phaseolus vulgaris* (cv. Black eye, Canario, Pinto 111, Red kidney, Sujinashi-edogawa, Taisho-kintoki, Carioca, Topcrop, Hon-kintoki, Kairyo-ohtebou, Master piece, Rico 23 and Rosinha), *Vigna unguiculata* cv. Kurodane sanjaku. All tested plants were grown from seeds sown in a sterilized mix of 9:1 clay loam to compost in small clay pots (94 mm opening × 83 mm height).

Leaves with mosaic and/or curl leaf (Fig. 1) were collected from passionfruit plant in different fiscal years in four districts: from a nursery shop in Akita City of Akita Prefecture in 2013, the Agriculture Research Center in Nago City of Okinawa in 2014 and a garden of a private house on Yoron Island and an agricultural field in Satsuma Town of Kagoshima Prefectures in 2015 (Fig. 2). To obtain a single isolate, four virus samples from Akita, Okinawa, Yoron and Satsuma districts were mechanically inoculated to healthy French bean (*P. vulgaris*) cv. Pinto 111 plants. Virus sap was extracted from diseased leaves in 0.025 M phosphate buffer (pH 7.0) using a mixture of 400- and 600-mesh carborundum. Two successive single-lesion transfers of Pinto 111 was used as inoculum sources because it was confirmed to be a suitable host for the single-lesion isolation of these viruses.

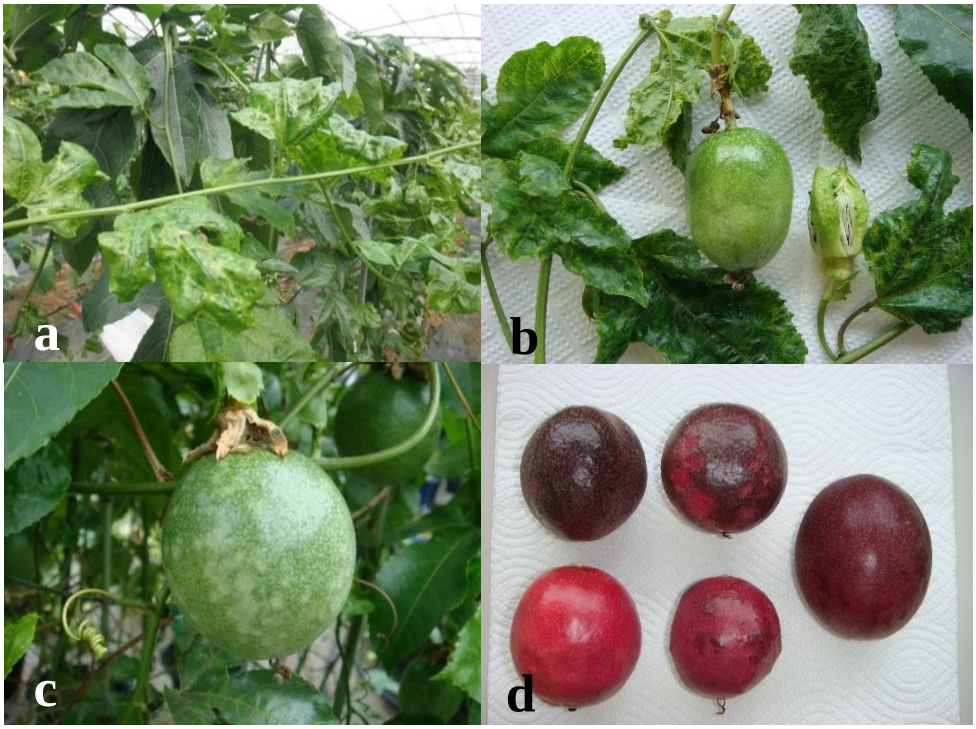


Figure 1. Characteristic foliar mosaic, leaf curl, and fruit malformation induced by isolate PV-OK on hybrid passionfruit (*Passiflora edulis* x *P.edulis* f. *flavicarpa*) at the site in Nago City (**a, b**) and again in the greenhouse at Kagoshima University (**c, d**). The fruit on the right side of plate **d** is healthy.

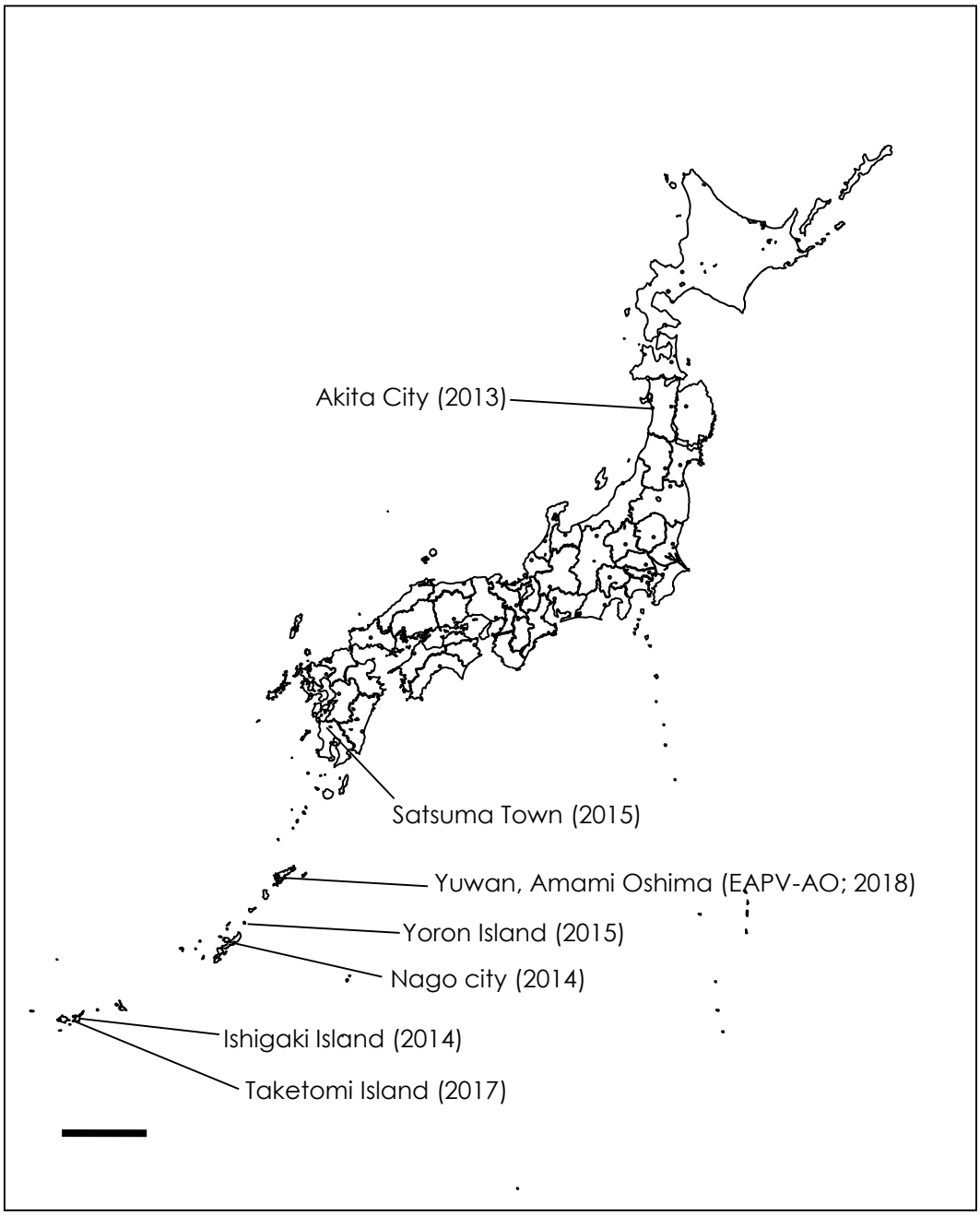


Figure 2. Collection locations and years of the isolates of new passionfruit viruses. The bar represents 200 km.

Each viral isolate was re-inoculated mechanically to a seedling of *P. foetida* with carborundum, in which the viruses had previously been shown to multiply systemically, causing leaf curl and mosaic symptoms on the upper trifoliate leaves (Fig. 3). A seedling of purple passionfruit (*P. edulis*) was also grafted inoculated using a stem from infected *P. foetida* optionally. These isolates were designated PV-AK (Akita), PV-OK (Okinawa), PV-YO (Yoron), and PV-SA (Satsuma) and were used for further study. Both infected *P. foetida* and purple passionfruit were used as virus-source hosts in a further experiment.

Another virus source used for these research experiments was the single isolate of EAPV-AO maintained in *P. foetida.* The isolate is belonging to Kagoshima University collection and was verified for their identities or species taxonomy by Reverse-transcription polymerase chain reaction (RT-PCR) described previously by Fukumoto et al. (2012).

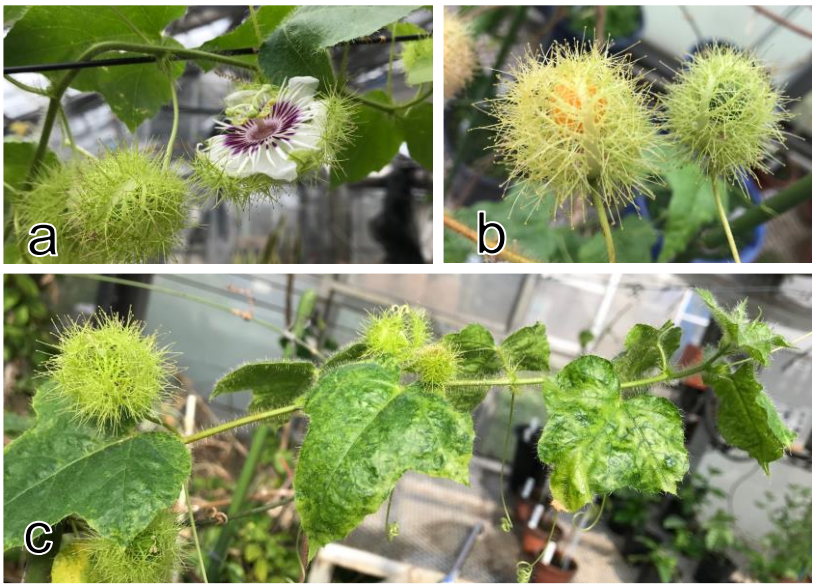


Figure 3. Aspects of *Passiflora foetida*. a Flower and calyx-covered immature fruits of *P. foetida* (healthy seedling), b Ripe and ripening fruit of *P. foetida* (healthy seedling), c Tip of *P. foetida* infected with EAPV-AO (10 months after inoculation).

All virus isolates and the plants materials for the experiments were kept in an insect-free green house at Kagoshima University (Korimoto, Kagoshima 31.5719^{o}N, 130.5452^{o} E), where the temperature was controlled at 18–36^{o}C under 12 to 16 hours photo periods.

4.2. Host range assay

Sap from *P. foetida* leaves infected with one of the four PV isolates or EAPV-AO in 0.025 M phosphate buffer (pH 7.0) was used to mechanically inoculate 22 cultivars or weed plants belonging to ten species of seven plant families. Hybrid passionfruit (Summer Queen) and purple passionfruit were not infected after mechanical inoculation with PV isolates and EAPV-AO in the preliminary experiment. Therefore, both viruses were inoculated by grafting scions of infected *P. foetida* to the clonal hybrid passionfruit (3 months after the cutting was planted) or to the purple passionfruit (4 months after seeding). In particular, since different cultivars of French bean (*P. vulgaris*) react differently to potyviruses (Chiembsombat et al. 2014; Wylie and Jones 2011), French bean was speculated as a suitable host for distinguishing the virus species. In this experiment, six plants of each cultivar and weed plant were inoculated, then evaluated up to 30 days or local and systemic infections caused by the viruses. Presence of the virus in symptomless leaves was examined by RT-PCR using the primer set of PV-F5 and PV-R7 (Table 1).

4.3. Electron microscopy

Virus-infected leaves, PV-OK isolate, from *P. foetida* was partially purified from 10 g of newly developed leaves 3 weeks after inoculation. The leaves were macerated in 1.5 volumes of extraction buffer 1/4 (w/v) 0.3 M potassium phosphate buffer, pH 7.6

containing 0.1% thioglycolic acid, 0.01 Na-DIECA, 8% chloroform and *n*-butanol and was filtered through two layers of cheesecloth. The crude sap was centrifuged at 8,500xg for 10 minutes in a Kubota RA-3F rotor. The supernatant was homogenized in 6% PEG and 0.1M NaCl and stirred at room temperature for 20 minutes. After stored at 4^{o}C for 1 hour, the supernatant was centrifuged as above procedure. The pellet was re-suspended two times in 0.05M borate buffer, pH 8.0 and differential centrifugated for 1 hour at 80,000 x g in a HITACHI S110AT angle rotor. The partial purified virus preparation was stored in a refrigerator (4^{o}C) until use. Examination of partial purified virus was made by negative staining with 2% phosphotungstic acid (pH 7.0) and observed with a HITACHI H7000KU electron microscope at 80 kV.

4.4. RNA extractions, reverse transcription-polymerase chain reaction and sequencing

4.4.1. RNA extractions

Total RNA virus was extracted from 0.2 g of the infected leaves of *P. foetida* or purple passionfruit as described by Dellaporta et al. (1983) with some modification. The fresh leaves were homogenized with 600 µl of 2 × STE (0.1 M NaCl, 0.05 M Tris–HCl, 1 mM EDTA), 60 µl 10% SDS, 6 µl mercaptoethanol and 600 µl of phenol–chloroform premixed with isoamyl alcohol (PCI, 25:24:1 v/v) followed by centrifugation at 15,000 rpm at 4 °C for 5 min. Then, 400 µl of supernatant containing nucleic acid was mixed with 1000 µl of 99.5% ethanol, followed by centrifugation at 15,000 rpm at 4 °C for 10 min.

The precipitate containing nucleic acids was dissolved in 400 µl of 1 × STE containing 35% ethanol, then 10 mg of CF-11 cellulose powder (GE Healthcare Japan)

was added. The suspension was then centrifuged as before. The resulting precipitate was resuspended in 400 μl of 1 × STE and centrifuged at 15,000 rpm at 4 °C for 1 min. The supernatant was then transferred to a new tube and ethanol precipitated. The pellet was dried under a vacuum and used as a template for reverse transcription (RT) after being dissolved in 20 μl of diethyl pyrocarbonate (DEPC)-treated water.

4.4.2. RT-PCR analysis

The first single-strand cDNA was synthesized with Oligo-dT primer using ReverTra Ace (Toyobo, Osaka, Japan) at 42 °C for 20 min and 99 °C for 5 minutes. PCR was performed using universal primers for potyvirus (NIb-F and D1000 modified) designed as a sense-primer based on the NIb-coding region (Zheng et al. 2008) and an anti-sense primer based on the CP-coding region (Langeveld et al. 1991) (Table 1, Fig. 4). The following five cDNA fragments encoding nearly the whole polyprotein were amplified using Ex-Taq polymerase (TaKaRa Bio, Shiga, Japan) and primers sets based on the alignment of full genomic sequences of potyviruses in the bean common mosaic virus (BCMV) subgroup (Adams et al. 2005b) (Table 1, Fig. 4). For ensuring that the fragments were derived from the same genome, the primers overlapped by at least 100 bp. The thermal cycler conditions were denaturation at 94 °C for 1 min; 35 cycles of denaturation for 30 s at 94 °C, primer annealing for 1 min at 56 °C and extension for 90 s at 72 °C; and final extension at 72 °C for 10 min.

Table 1. Primers used in obtaining the complete sequences of PV isolates

Primer	Sequence 5′- 3′	Position (nt)
PV-R7	CCCATACCAAGTAATGTGTG	9,748－9,767
PV-F7	CTACATGCCTAGGTACGGAC	9,510－9,529
PV-F6	ATGCACCACTTCTCAGACGC	9,451－9,470
D1000 modified	GTNCCRTTNTCNATRCACCANAYCAT	9,319－9,344
PV-F5	ACTCAATGCTGGGGACGCTC	8,967－8,986
PV-RT5	ATATGTGAGTCCAAGTTCCG	8,513－8,532
PV-R6	CCATTCATCCTTCTCCTCTG	8,465－8,484
NIb-F	TNTGYGTNGAYGAYTTYAATAA	7,996－8,018
PV-RT4	CGACATCAAGGTATTTAGCC	7,223－7,243
PV-R5	GCCCACTATATGACCATCAG	7,139－7,158
PV-F4	GAAYAGCAATGCNATAG	6,546－6,562
PV-RT3	ACTTCATTGTAGCTTGCCAC	4,795－4,814
PV-R4	TGCCGATACTTTGAGTAGCT	4,625－4,644
PV-F3	CGTGGGATCTGGAAAATCAAC	4,290－4,310
PV-RT2	TCTCTGATCGACTGTGCCTC	2,834－2,853
PV-R3	CCTTGGTAGTTCAGCATTCC	2,660－2,679
PV-F2	CATGGCCAACAATGACTGAC	2,594－2,613
PV-RT1	ATGCATGGTACCTCCACTTC	505－524
PV-R2	CCTTGCTGTGCATCCTATTG	275－294
PV-R1	TCAAGCCTGGTAGTTTCCAC	205－224
PV-F1	GGTGGAAACTACCAGGCTTG	204－223
Oligo-dT	TTTTTTTTTTT	-
10×UPM	CTAATACGACTCACTATAGGGCAA GCAGCAGTGGTATCAACGCAGAGT	-
Univ Short Primer	CTAATACGACTCACTATAGGGC	-

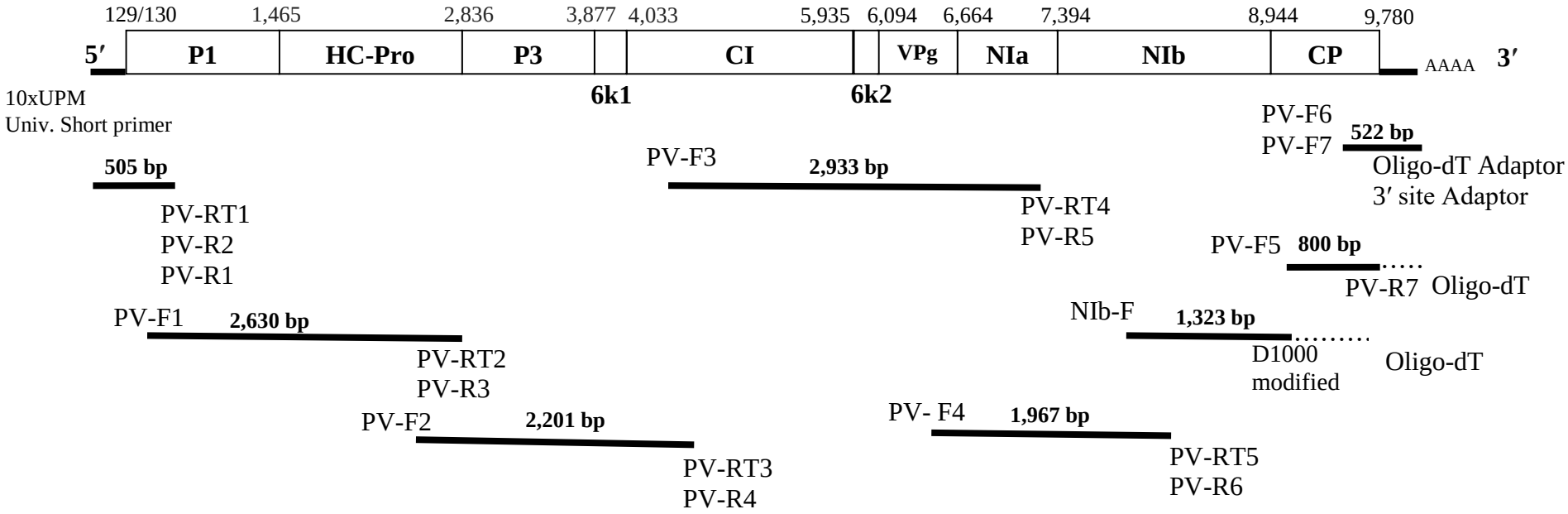


Figure 4. RT-PCR amplification strategy for obtaining cDNA encoding complete genomes of PV isolates from infected *P. foetida* or purple passionfruit leaves. The NIb coding region was first reverse transcribed (RT) with oligo-dT primer and PCR amplified using potyvirus universal primers pair NIb-F/D1000 modified. Five cDNA fragments were synthesized with PV-RT2, -RT3, RT4, -RT5, -R7 and PCR amplified using primers pairs as PV-F1/PV-R3 to PV-F5/PV-R7 that constructed from BCMV subgroup conserved region. The 5' NCR was RT with 5'-phosphorilated-RT primer and SMARTER II oligonucleotide followed by nested PCR amplification using first primers pair 10xUPM/PV-R2 and second primers pair universal short primer/PV-R1. The 3'NCR was RT using oligo-dT Adaptor primer and PCR amplification using first primers pair PV-F7/3'site adaptor.

For obtaining a fragment of the 5'-end noncoding region (NCR), the cDNA was synthesized by using SMARTScribe Reverse Transcriptase of a 5′ SMARTER RACE 5/3 kit (TaKaRa) with known genes containing 5'-phosphorylated-RT primer (RV-RT1) and SMARTer II Oligonucleotide and the manufacturer's instructions. The cDNA product was PCR-amplified by KOD-plus Neo DNA polymerase (Toyobo) using a universal long primer (10XUPM) and gene-specific primer (PV-R2), followed by nested PCR using a universal short primer and gene-specific primer (PV-R1) (Table 1, Fig. 4) and the thermal cycling conditions are pre-denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 15 sec, annealing at 58 °C for 30 sec, and extension at 68 °C for 1 min/kb.

For obtaining a fragment of the 3' NCR, total RNA was reverse transcribed using the Oligo-dT Adaptor primer from the 3' full RACE Core set (TaKaRa) and the manufacturer's instructions. The cDNA product was synthesized with the upstream known CP sequence primers (PV-F6 or -F7) and the 3' site Adaptor Primer (Table 1, Fig. 4) using TaKaRa LA-Taq polymerase (TaKaRa) with thermal cycling as follows: one cycle of 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 68 °C for 30 s; and final extension for 5 min at 72 °C.

PCR amplicons, fragment for coding protein, 3'- and 5'-NCR products were visualized on 1.0% DNA agarose gel by electrophoresis. The gels run with 5 µl of PCR product and 1 µl of loading buffer. Tris-acetate acid electrophoresis buffer was used for gel and run buffer (50xTAE: 2M Tris-acetate; 0.05 M EDTA; pH 8.0 Sambrook et al. 1989). The molecular marker weight was used for standard size are 100bp DNA ladder. The gel was run on Bio-Rad Mini-Sub Cell™ or Bio-Rad Wide Mini-Sub Cell™ at 100 V for ± 25 min and product was separated. Gel were stained in a 1 µg/mL EtBr solution for 8 min. The gel was visualized on a trans-illuminator.

4.4.3. Sequences analysis

Each amplified fragment for coding protein and 5’ NCR product was purified using the QIA quick Gel extraction kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. The purified DNA amplicons were direct sequenced or cloned into T-vector pMD20 (Novagen, Merck, Darmstadt, Germany) and pBluescript II SK (+) (Agilent, Santa Clara, CA, USA) plasmid using T4DNA Ligase enzyme (TaKaRa) and transformed into *Escherichia coli* strain XL-1 Blue High Efficiency Competent Cells (Agilent). Transformed DNA products were cultured in Luria broth (LB). The recombinant cDNA clones were extracted using an Illustra plasmid Prep Mini Spin kit (GE Healthcare Life Sciences, Marlborough, MA, USA). The clones of the 5’ NCR and the polyprotein-coding region were sequenced using a specific T7 or M13M4, and M13RV flanking vector primer, and specific primers (Table 2), respectively. The full 3’ NCR sequence was obtained by direct sequencing using the specific PV-F7 primer (Table 1, Fig. 4). The products were extracted in a solution contains 99.5% ethanol (62.5 µL), sterile water (24.5 µl), 3M sodium acetate (3 µl). After for 20 min incubated in room temperature, the products were centrifuged at 14.000 rpm for 20 min. The pellet was washed in 70% ethanol and centrifuged for 5 min at 14.000 rpm. The DNA pellet was dissolved in 20 µl formamide. Nucleotides were sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

The assembled sequences of each complete coding region were compared to the related sequences using the BLAST algorithm with GenBank database (Altschul et al. 1990). The assembled sequence alignments and pairwise comparisons of nucleotide and amino acid identities against the cognate species were calculated using the GENETYX

6.0 program and the needle method in the EMBOSS pairwise alignment algorithms program (http://www.ebi.ac.uk/Tools/psa/emboss_needle), respectively. Before constructing a phylogenetic tree, we examined recombination breakpoints in the polyproteins using the RDP, GENECONV, BOOTSCAN, MAXCHI, and SISCAN algorithms and the 3SEQ method as implemented in RDP 4 (Martin et al. 2015). Phylogenetic relationships among the isolates were analyzed using the neighbor joining and the maximum likelihood methods as implemented in MEGA ver. X program (Kumar et al. 2016) and bootstrapping with 1000 replicates.

Table 2. The direct sequencing primer used in obtaining the complete sequences of PV isolates

Primer	**Sequence 5′- 3′**	**Position (nt)**
TNJ-DiSeq-3'NCR-F	ACTACATGCCTAGGTACGGA	9,520－9,510
TNJ-DiSeq-R1	GAGATGCTAACTGTGGAAAG	9,814－9,794
TNJ-DiSeq-R2	GTTGCTCTGGTGTTGAATAG	9,212－9,231
TNJ-DiSeq-R3	ATATGTGAGTCCAAGTTCCG	8,514－8,534
TNJ-DiSeq-R4	CGACCATGAGGGTGTTATC	8,346－8,363
TNJ-DiSeq-R5	ATCAACTGCCTTCTCAAAGG	7,659－7,678
TNJ-DiSeq-R6	AACAATACACGCACGCTCCT	6,981－7,000
TNJ-DiSeq-R7	CTCAGAGTCGTATAGTTCTC	6,299－6,318
TNJ-DiSeq-R8	GCCTGCGATTGCATTACTAT	6,549－6,568
TNJ-DiSeq-R9	CGAACAGCATTTAGCACACC	5,909－5,929
TNJ-DiSeq-R10	AGCTCGAAGTTTAGCGCACT	5,281－5,300
TNJ-DiSeq-R11	GGTGTTGCCGATACTTTGAG	4,650－4,631
TNJ-DiSeq-R12	GAACTCTGACTTCTTCACCG	4,012－4,032
TNJ-DiSeq-R13	TTCCAACAAGCTTAGTGCGC	3,327－3,346
TNJ-DiSeq-R14	TTCCAACAAGCTTAGTGCGC	3,327－3,346
TNJ-DiSeq-R15	GATTTGCTGTTCCTGCCTTC	2,758－2,779
TNJ-DiSeq-R16	TCGAGAAGAACCGCTTTGAG	2,107－2,127
TNJ-DiSeq-R17	TGGTGGGAGTAGTGTTCAAT	1,454－1,447
TNJ-DiSeq-R18	GTCTGCTTAACAGGACTTTG	784－804

4.5. Dual infection analyses of EAPV and EAPDV

4.5.1. Virus collection, RNA isolation and RT-PCR

In order to clarify the starting point of the experiment in this chapter, twenty-one passionfruit plants which are assumed to be virus-infected were collected, from several places in Kagoshima and Okinawa prefectures, and detected pathogenic viruses. To detect presence of virus, samples were extracted as described in the method of first experiment. Five hundred nanogram of total RNA was reversed transcribed with oligo-dT primer using ReverTra Ace (Toyobo, Osaka, Japan) at 42 °C for 20 min and 99 °C for 5 minutes. The cDNA was amplified using specific primers pair of EAPV and EAPDV (Table 3). The thermal cycler conditions were at 94 °C for 1 min for initial denaturation, 35 cycles for 30 s at 94 °C for denaturation, 1 min at 56 °C and extension for 90 s at 72 °C for primer annealing and at 72 °C for 10 min for final extension. PCR product were visualized on 1.0% DNA agarose gel by using electrophoresis with 100bp DNA ladder for standard size and the gel was run on Bio-Rad Mini-Sub Cell^{TM} at 100 V for about 20 min. Gel was stained in 1 µg/mL EtBr solution for 8 min and visualized on a transilluminator.

4.5.2. Virus, pre-inoculation and inoculation.

Virus sources used for the dual infection assay are a single isolate of EAPV-AO and EAPDV-OK, proposed name for the identical PV-OK, both belonging to the Kagoshima University collection, the identity or species taxonomy of each was verified by reverse-transcription polymerase chain reaction (RT-PCR) as described previously (Fukumoto et al. 2012; Riska et al. 2019). *P. foetida* infected with the isolates was dehydrated and stored on calcium chloride in a glass container. Then, prior to the experiment, they were ground in 0.025 M sodium-phosphate buffer (pH 7.0) (SPB) and

mechanically back-inoculated into healthy *P. foetida*, and the plants were kept in an insect-free greenhouse at Kagoshima University (Korimoto, Kagoshima 31.5719^{o}N, 130.5452^{o} E), where the temperature was controlled at 18-36^{o}C under 12- to 16-hour photo-periods.

At the beginning of the experiment, the primary leaves of *Phaseolus vulgaris* cv. Pinto 111 were mechanically inoculated with the ground solution of *P. foetida* infected with each virus. A newly developed leaf of a healthy *P. foetida* plants was mechanically inoculated with EAPV and EAPDV obtained from local lesions at the sixth to seventh-leaf stage, equivalent to about 60 days after germination. The plants were verified to be virus-free by RT-PCR before inoculation with EAPV or EAPDV. The newly developed leaves that showed symptoms 10 days after inoculation (dai) were used as the inoculum for this experiment, and the amount of proliferated EAPV (in terms of nucleic acid) measured by RT-PCR analysis in preliminary experiment was constantly 2.0-2.4 times higher than that of EAPDV (details methods are described later). Therefore, the inoculum was prepared by grinding *P. foetida* leaf at a leaf : buffer ratio of 1 : 20 (w : vol). After centrifugation of the leaf crude extract at 8,500xg for 5 min, the supernatants were diluted with SPB and viral concentration was adjusted to the same level.

4.5.3. Experimental design and statistical analysis.

To study the interaction of EAPV and EAPDV in *P. foetida*, the experiment was carried out with five different treatments and 5 plants for replications described as follow. In the three of the five experimental plots, plants were inoculated only once (time 0) as follows: inoculation with EAPV (A); inoculation with EAPDV (D); and co-inoculation with a mixture of EAPV and EAPDV (A+D). For the co-inoculation, the same amount of

each virus inoculum was mixed at 1:1 (v/v) ratio. The inoculum was applied by rubbing the third and fourth expanded leaves from the top of *P. foetida* in the sixth to seventh-leaf stages using SPB, pre-dusted with carborundum (600 mesh). In the remaining two plots, the plants received a second inoculation 6 days after the first inoculation (two step treatment). On the second inoculation, the inoculum was applied to the fifth and sixth leaves from the top, those meaning the leaves just above the first inoculated ones. The two-step treatments are: first with EAPDV and then followed by EAPV (D→A) and first with EAPV and then followed by EAPDV (A→D). At 2 to 3 min after the inoculation, surface of the treat leaves was washed with running water and the plants were kept in insect-free greenhouse at 18 to 35ºC. The viral concentration in inoculated leaves was calculated at 1 day after first and second inoculations, and those non-inoculated upper leaves almost every week up to 28 dai using RT-qPCR described later.

The data presented in Figure 11 and 13 are standard deviation means of three and five replicates, respectively. The statistical evaluation was determined by analysis variance (ANOVA) using Prog. Minitab ver. 19 (Pennsylvania, USA). Significant effects were further analyzed with Tukey's HSD at $p<0.05$.

4.5.4. Evaluation of virus symptoms

The severity of symptoms was calculated according to four points scales based on viral damage described by us (Fig. 11) as follows: 1= symptomless, 2= slight mosaic, 3= mosaic and mottling, 4= severe mosaic and mottling. The observation was measured at almost every week until 28 dai.

4.5.5. Analysis of virus accumulation and gene expression by real time polymerase chain reaction (RT-qPCR)

100 mg leaf tissue from the inoculated leaf (at 1 dai) and the third leaf from the youngest shoot (at every week) were collected. Total RNA was ground in liquid nitrogen and extracted according to the protocol of NucleoSpin® RNA Plant kit (Macherey-Nagel GmbH&Co.KG). The RNA quantity and quality were determined spectrophotometrically using Biospec-mini DNA/RNA/protein analyzer (Biotech, Shimadzu) by measuring absorbance ratios.

Five hundred nanograms of complementary DNA was synthesized using Rever-Tra Ace (Toyobo) with oligo-dT primer at 42ºC for 20 min and at 99ºC for 5 min. PCR was carried out using Ex-Taq polymerase (TaKaRa Bio, Shiga, Japan) and specific primer set for EAPV and EAPDV (Table 3). The thermal cycler condition as described above methods. The cDNA fragment of each virus was used as the source of standard targeting genes in real time polymerase chain reaction (RT-qPCR) analyses.

The sequences in the GenBank database were used in designing the primers (Table 3), as well as sequences obtained in this study. The primers for EAPV targeting 159-bp amplicon size of CP coding region were designed from the region at 8,894 nt and ending at 9,052 and the primers for EAPDV targeting a 203-bp amplicon CP coding region were 8,967 nt to 9,179 nt. The primers for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Acc. LC462273) were designed from the genome of *Passiflora alata* (AY858258). The 670-bp cDNA fragment of *P. foetida* was used for targeting the 110-bp amplicon of the standard *GAPDH*. This gene was used to normalize the cDNA level of gene targeting.

Table 3. Nucleotide sequences of forward and reverse primers used for virus amplification and quantitative reverse-transcription PCR of RNAs of EAPV, EAPDV, Pathogenicity Related genes (PR-gene) and *GAPDH* gene

Primer	Nucleotide sequence (5'- 3')	Position (nt)	Purpose	Accession
EAPV-SF	CGACGAATCCAAATCCCAAT	8,894-8,913	RT-PCR and RT-qPCR	AB690454
EAPV-R	CGAGTATGACCCTACCTTTC	9,033-9,052		
EAPV-SR	ATGTTCTGATTGACATCCCG	9,609-9,628		
EAPDV-SF	ACTCAATGCTGGGGACGCTC	8,967-8,986	RT-PCR and RT-qPCR	LC373083
EAPDV-R	CAAGTACAATTCTACC AGCA	9,159-9,179		
EAPDV-SR	CAGAACCCATACCAAGTAAT	9,763-9,782		
LOX-qF	AAGCCGGAAGACGCTTTGC	-	RT-qPCR	GQ141712
LOX-qR	CTCTTCATCAGGTGAATGG	-		
Cysteine-qF	ACCAGGATGAGAACAGCAA	-	RT-qPCR	MF581089
Cysteine-qR	AGATGGTTGTGATGGAGGGT	-		
GAPDH-qF	TACTGGAGCTGCTAAGGCTG	-	RT-qPCR	AY858258
GAPDH-qR	TCAAGTCTCACAGTGAGGTC	-		

The other gene of interest are two type of pathogenesis related (PR) genes such as lipoxygenase-2 (*LOX2*) and cathepsin B-like cysteine protease. The primers of each gene were chosen from a conserved region of *P. edulis* accession GQ141712 and MF581089, respectively. Those regions were compared to the other related plants to obtain the *LOX2* (Acc.LC458799) and cathepsin B-like cysteine protease genes (Acc.LC462275) of *P. foetida*, respectively. Fragments of *LOX2* (102 bp) and cathepsin B like-cysteine protease gene (115 bp) were amplified.

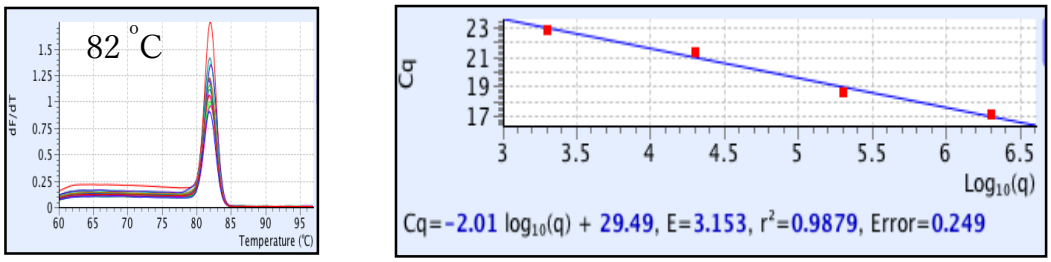
The real time PCR was done to find out the titer of EAPV, EAPDV and accumulation of *GAPDH* gene observed at 1, 14, and 28 dai in leaf tissue. Accumulation of *LOX2* and cathepsin B-like cysteine protease were taken at 14 dai. Each sample was subjected to reverse transcriptase as the protocol for virus detection, as explained above. The total PCR reaction mix of 10 µl consisted of 1 ng/µl cDNA template, 5µM of TB green as reference dye, and 0.4µM of specific primer pair for each gene and was applied to a Light-Cycler 480 Real-time PCR system (Roche Diagnostics GmbH, Penzberg, Germany). The standard curve of the targeting genes was generated from the linearized CP of EAPDV and EAPV, *GAPDH*, *LOX2* and cathepsin B-like cysteine protease genes. The purified fragments (1 ng/µl in 20 µl) obtained from RT-PCR amplification with specific primer of each virus and genes (Table 3) were 5 diluted serially in water to obtain 10^2 to 10^6 copies number. The copies number of each DNA template was calculated based on the following formula: (amount x $6.022x10^{23}$)/ (length x $1x10^9$x 650). The dilutions were used to calculate a linear regression of the unknown amount of viral genome cDNA and fluorescence value (Cq) of RT-qPCR.

The amplification protocol was run at: 95^{o}C for 2 min, 95^{o}C for 10 min, 55^{o}C

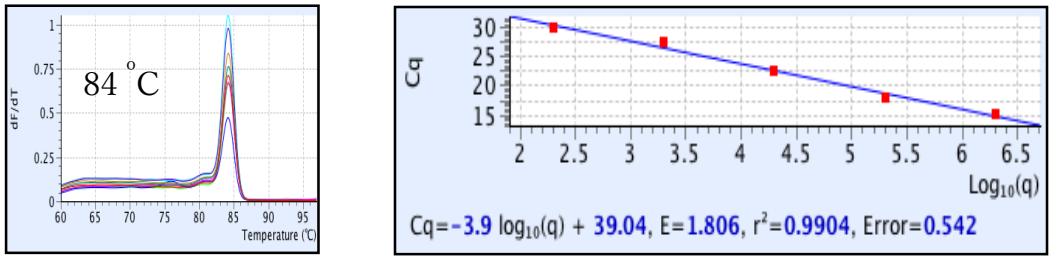
for 10 s, 72ºC for 15 s for 45 cycles, and melting curve from 55 ºC to 95ºC with 0.5ºC increments for 15 s. Gene-specific amplification of both viruses, *GAPDH*, *LOX2* and cathepsin B-like cysteine protease genes were confirmed by the appearance of a single, dominant peak in the RT-qPCR dissociation curve analyses (Fig. 5). The relative viruses and gene accumulation ratio (AR) were calculated as follow:

$$AR=\frac{\text{virus/gene quantity means}}{\text{GAPDH quantity means}}$$

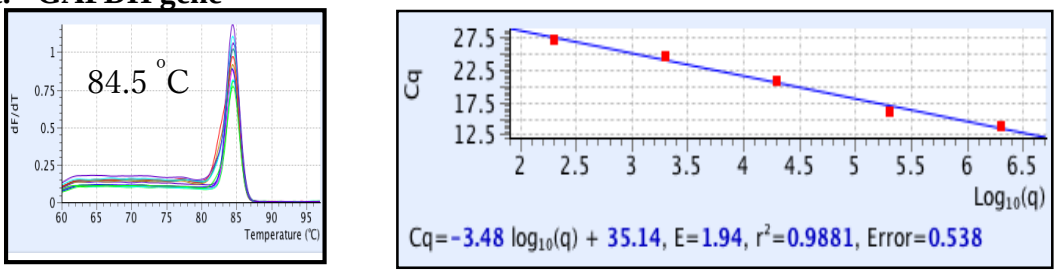
a. EAPV



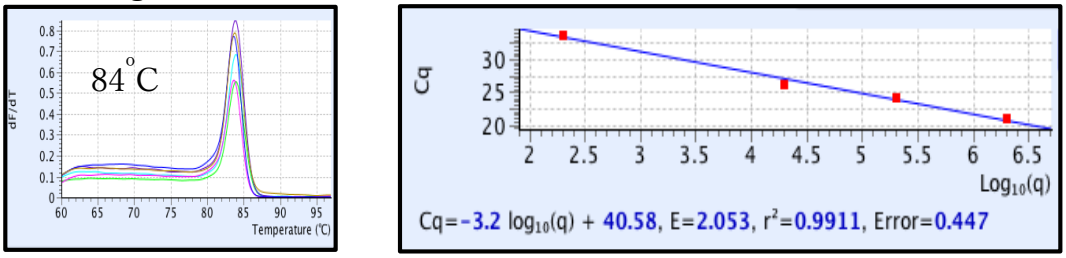
b. EAPDV



c. GAPDH gene



d. *LOX2* gene



e. Cathepsin B like-cystein protease

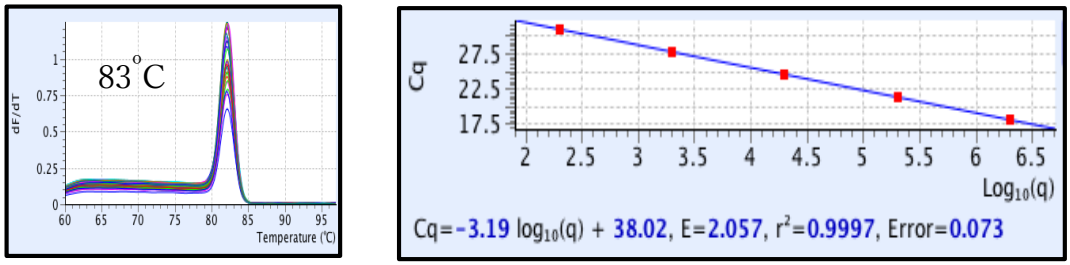


Figure 5. Melting temperature analysis and standard curve plot using CP of EAPV, EAPDV, *GAPDH*, *LOX2* and Cathepsin B like-cysteine protease genes in RT-qPCR.

V. RESULTS

5.1. Biological and molecular detection of a novel potyvirus species causing deformation of passionfruit in Japan

5.1.1. Host range and symptomatology

In general, the 12 species belonging to seven plant families reacted variously after inoculation with the four PV isolates and EAPV-AO (Table 4). In several plants, infection was restricted to a local infection of the inoculated leaves. Purple passionfruit and hybrid passionfruit inoculated with the PV isolates developed the same foliar symptoms as passionfruit in the field and eventually deformed fruits (Fig. 1). The reaction of *N. benthamiana* to the PV isolates differed from that to EAPV-AO; the four PV isolates caused a symptomless systemic infection whereas the timing and the presence or absence of mosaic after EAPV-AO inoculation varied on the upper non-inoculated leaves. The reaction of French bean (*P. vulgaris*) to the viruses varied by cultivar, but four cultivars were infected systemically by PV isolates rather than by EAPV-AO. However, in the case of the French beans, no matter which cultivar was used, it was difficult to distinguish between the PV isolates and EAPV-AO visually in the early stage after inoculation. Notably, the reaction of cowpea (*V. unguiculata*) cv. Kurodane sanjaku to infection by the PV isolates clearly differed from that to infection by EAPV-AO. Kurodane sanjaku was infected systemically by the PV isolates, and local lesions on the inoculated leaves appeared just one week after inoculation, while the same cultivar seemed to be immune to EAPV-AO.

Table 4. Host range and foliar symptoms caused by the PV isolates and EAPV-AO on inoculated (Inoc.) leaves and non-inoculated (Non.) upper leaves

Plant hosts	PV-AK, PV-OK, PV-YO, PV-SA		EAPV-AO	
	Inoc.	Non.	Inoc.	Non.
Capsicum annuum cv. California wonder	-	-	-	-
Chenopodium amaranticolor	LL	-	LL	-
Chenopodium quinoa	Lat	-	LL	-
Datura stramonium	-	-	-	-
Nicotiana benthamiana	Lat	Lat	Lat	Lat or Mo
Nicotiana glutinosa	-	-	-	-
Nicotiana tabacum cv. Bright Yellow	Lat	-	-	-
Passiflora edulis	na[a]	LC, Mo[a]	na[a]	LC, Mo[a]
Passiflora edulis x *P. edulis* f. *flavicarpa*	na[a]	LC, Mo[a]	na[a]	LC, Mo[a]
Passiflora foetida	LL	LC, Mo	Lat or LL	LC, Mo
Phaseolus vulgaris				
cv. Black eye	Lat	Lat	-	-
cvs. Canario, Pinto 111, Red kidney, Sujinashi-Edogawa, Taisho-Kintoki,	LL	-	LL	-
cvs. Carioca, Topcrop	-	-	-	-
cvs. Hon-Kintoki, Kairyo-Ohtebou	LL	LC, SN	LL	-
cv. Masterpiece	LL	Lat	LL	-
cvs. Rico 23, Rosinha	LL	-	LL	LC, SN
Vigna unguiculata cv. Kurodane sanjaku	LL	Mo	-	-

LL local lesion, - no infection (as confirmed by RT-PCR), *Lat* latent infection (as confirmed by RT-PCR, na not applicable (graft-inoculation), *LC* leaf curl, *Mo* mosaic, *SN* systemic necrosis.

[a] Viruses were graft-inoculated using scion of infected *P. foetida.*

5.1.2. Electron microscopy

Flexuous, filamentous particles were observed by electron microscopy in negatively stained partially purified preparation (Fig. 6). As a result of measuring the length of 20 particles, the average length was 810 nm with the standard deviation of 17.7 nm. The length of the measured virus particles was within the range of 780–854 nm.

5.1.3. Sequence properties of the isolates

The complete nucleotide sequences of PV-AK (DDBJ accession LC379162), PV-OK (LC373083), PV-YO (LC377302), and PV-SA (LC375413) are almost identical. The genome comprises 9973 nucleotides (nt) with 129–130 nt of the 5’ NCR and 193 nt of the 3’ NCR excluding the poly(A) tail. The open reading frame (ORF) of the isolates starts with AUG located at 130–132, except for PV-AK, which starts at 131–133 and terminates at codon UAA located at 9778–9780. The start code GAAU for the 5’ NCR is a conserved sequence among the isolates except for PV-AK (AAAU). The isolates contain a single putative ORF that encodes a polyprotein of 3217 aa residues with a relative molecular mass of approximately 366.64 kDa. The ORFs encodes 10 proteins: nucleotides 130–1464 (P1), 1465–2835 (HC-Pro), 2836–3876 (P3), 3877–4032 (6KI), 4033–5934 (CI), 5935–6093 (6K2), 6094–6663 (VPg), 6664–7393 (NIa), 7394–8943 (NIb), and 8944–9780 (CP).

Typical for potyviruses, the polyprotein of the four PV isolates has several conserved motifs, which were identical in the polyprotein-coding region for all the PV isolates. A conserved motif on P1, which has proteinase activity and a structural aspect present as GDSG and FIXRG instead of the common GWSG and FVXRG. HC-Pro has

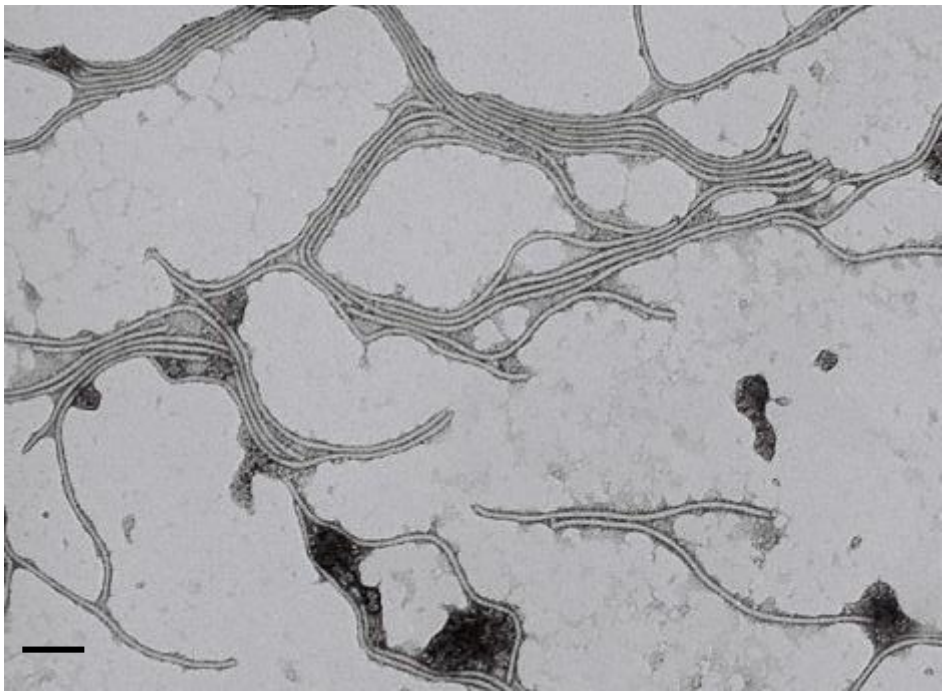


Figure 6. Electron micrograph of PV-OK particles. The sample was partially purified and negatively stained with 2% phosphotungstic acid. The bar represents 200 nm

the KLSC, FRNR, and IDS instead of the most classical motifs, i.e., the KITC, FRNK and IGK motifs, which are associated with aphid transmission, expression of symptoms, and an RNA silencing suppressor (Gal-On 2000; Shiboleth et al. 2007).The cylindrical inclusion (CI) of the PV isolates contains the GAVGSGKST motif, which plays roles in NTP binding and helicase activity, as found in lettuce Italian necrotic virus (Ciuffo et al. 2016), and the DECH motif in typical helicase superfamily members as found in pecan mosaic-associated virus (Fernández et al. 1997; Su et al. 2016).

The PV isolates also have three conserved motifs (i.e., ERIQRLGRVGR, ATNIIENGVTL and KVSAT) that might be involved in RNA binding or unwinding and ATP hydrolysis (Adams et al. 2005a; Riechmann et al. 1992). The VPg protein contains NMYG motifs present as HMYG for a viral RNA attachment function. The NIa proteins of PV isolates have H, D, and C at the same positions known to participate in protease cleavage (Dougherty et al. 1989). The NIb protein has the CHADGS motif (which is also expected to be involved in putative RdRp activity) and SGX3TX3NTX30GDD, which is a conserved motif in viral RdRp (Domier et al. 1987). The PV isolates have DVG motifs instead of DAG motifs in CP proteins, where the motif is exposed in the N-terminal region of the CP (Urcuqui-Inchima et al. 2001).

A minor change was identified in the PV isolates at the five cleavage sites of PI/HC-Pro (IEHY/S, different amino acid residues are underlined), HC-Pro/P3 (YRVG/G), CI/6K2 (VRLQ/S), 6K2/VPg (VTTQ/G), and NIb/CP (VSLQ/S), respectively, compared with the other isolates in potyvirus groups (Table 5).

Table 5. Comparison of the PV isolates cleavage sites within different potyvirus isolates.

Protein junction	PV-AK, PV-OK, PV-YO, PV-SA	EAPV-AO	EAPV-IB	BCMV	BCMNV	CABMV	PWV
P1/HC-Pro	IEHY/S	VVHY/S	VQHY/S	IHHY/S	IEHY/T	VQHY/S	IHHY/S
HC-Pro/P3	YRVG/G	YKVG/G	YRVG/G	YRVG/G	YRVG/G	YKVG/G	YRVG/G
P3/6K1	VATQ/S	VSCQ/A	VTCQ/A	VSVQ/A	V(G)DTQ/A	GRHQ/A	VELQ/A
6K1/CI	VRVQ/S	VKIQ/S	VKVQ/S	VQM(I)Q/S	VRPQ/S	VRVQ/G	VKVQ/S
CI/6K2	VRLQ/S	VQLQ/S	VQLQ/S	VRLQ/G(S)	VRLQ/G	VCLQ/S	VRLQ/S
6K2/VPg	VTTQ/G	VSTQ/G	VVTQ/G	VT(A)TQ/G	VSTQ/G	VTTQ/G	VSTQ/G
VPg/NIa-Pro	VQTE/S	VELE/S	VELE/S	VA(D)V(I)E/S	VELE/S	VGVE/S	VELE/S
NIa-Pro/NIb	VTTQ/S	VVVQ/S	VIVQ/S	V(L)A(E)TQ/S(I)	VSVQ/S	GCTQ/S	VSTQ/S
NIb/CP	VSLQ/S	VSLQ/T	VSLQ/S	VHLQ/S	VSTQ/S	VV(M, R)LQ/S	VSLQ/S

PV-AK Passiflora virus-Akita, *PV-OK* Passiflora virus-Okinawa, *PV-YO* Passiflora virus-Yoron, *PV-SA* Passiflora virus-Satsuma, *EAPV-AO* East Asian Passiflora virus-Amami Oshima, *EAPV-IB* East Asian Passiflora virus-Ibusuki, *BCMV* bean common mosaic virus, *BCMNV* bean common mosaic necrosis virus, *CABMV* cowpea aphid-borne mosaic virus, *PWV* passionfruit woodiness virus

A major change was identified in other cleavage sites in P3/6KI (VATQ/S), 6KI/CI (VRVQ/S), VPg/NIa-Pro (VQTE/S), and NIa-Pro/NIb (VTTQ/S) (Table 5).

5.1.4. Comparison between the isolates and members of *Potyvirus*

The complete genome sequence of the four isolates analyzed with BLAST and GENETYX 6.0 analyses showed that they share 72–73% identity with bean common mosaic necrosis virus (BCMNV) (KY659304; percentage of query cover: 49%) followed by soybean mosaic virus (SMV) (KC845322; percentage of query cover: 56%) and PWV (HQ122652; percentage of query cover: 46%). According to our EMBOSS needle analysis, PV-YO shares only 65.9% nt and 66.6% aa with BCMNV (Table 6). The identity of the nucleotides at the 5' NCR and the 3' NCR shared with BCMNV was only 53.1% and 59.4%, respectively. The comparison of sequence identities among the PV-AK, PV-OK, PV-YO and PV-SA isolates (Table 6) indicated that the isolates shared up to 99.2–99.6% nt and 99.3–99.6% aa identity for the CP sequence, and up to 100% identity for the 6K1 sequence.

The sequence alignment comparison for CP aa identity at the N-terminal region revealed that one and two amino acids for PV-YO differed from those of PV-AK and PV-SA, respectively, and four amino acids were distinct from PV-OK. At the whole polyprotein level, the four isolates shared 98.4–98.5% nt and 98.4–98.9% aa identities. The complete nucleotide sequence identities between the PV-YO and other members of potyvirus ranged from 65.0 to 68.1%, and their complete ORFs shared 63.9–70.1% identity. PV-YO shared the highest nucleotide sequence identity with watermelon mosaic virus (WMV, DQ399708): 68.1% for the full genomic sequence level, 70.1% for the

Table 6. Nucleotide (nt) and amino acid (aa) sequence identities (%) between the PV-YO and potyviruses species including three PV isolates.

Virus isolates	FG Nt	ORF Aa	5'NCR Nt	P1 aa/nt	HC-Pro aa/nt	P3 aa/nt	6K1 aa/nt	CI aa/nt	6K2 aa/nt	VPg aa/nt	NIa-Pro aa/nt	NIb aa/nt	CP aa/nt	3'NCR nt
PV-AK (Akita)	98.4	98.9	90.4	97.5/98.0	98.5/97.5	99.1/99.1	100/100	99.5/98.3	98.1/99.4	99.5/98.6	98.4/97.9	99.0/98.7	99.6/99.2	100
PV-OK (Okinawa)	98.3	98.4	96.9	97.5/97.8	98.7/98.2	98.8/98.8	98.1/98.7	98.7/98.4	96.2/98.1	97.4/97.7	97.9/97.4	99.2/98.5	98.6/99.3	98.5
PV-SA (Satsuma)	98.5	98.7	97.7	97.3/97.8	98.5/98.1	98.6/98.8	100/100	99.2/98.6	98.1/98.7	99.5/98.2	97.1/97.9	99.8/98.8	99.3/99.6	99.5
BCMV (AJ312437)	66.3	65.6	75.0	41.6/56.6	77.7/67.1	45.8/60.9	61.5/63.7	75.0/70.2	58.5/67.3	76.8/72.4	70.8/68.9	72.9/68.2	72.1/71.5	53.2
BCMNV (U19287)	65.9	66.6	53.1	24.4/43.8	77.7/69.6	47.6/60.9	71.2/68.6	77.8/71.0	64.2/68.6	79.5/69.0	70.8/69.6	79.1/71.7	61.3/75.4	59.4
PWV (HQ122652)	65.0	63.9	47.9	26.0/44.0	72.0/68.6	43.5/57.9	69.2/68.9	76.5/69.9	58.5/62.1	72.6/65.7	70.4/69.3	76.8/69.9	75.9/70.1	53.8
SMV(S42280)	64.7	64.6	55.8	25.4/43.0	73.7/69.3	46.7/57.6	65.4/68.8	74.8/67.3	56.6/62.1	76.3/69.5	71.6/69.1	80.5/72.9	72.0/68.4	49.8
EAPV-AO (AB246773)	65.3	66.2	47.0	42.2/54.3	71.8/68.0	45.2/59.5	61.5/69.4	77.1/70.9	60.4/64.8	68.4/67.4	74.5/68.5	77.1/70.3	70.3/67.6	47.2
EAPV-IB (AB604610)	65.4	66.1	26.5	40.6/54.9	73.7/68.5	42.9/58.8	61.5/69.6	76.8/70.3	64.2/68.5	72.1/69.5	72.4/67.8	77.6/70.7	67.0/69.5	53.1
WMV (DQ399708)	68.1	70.1	60.0	57.9/65.7	75.9/71.6	46.9/59.7	73.1/73.2	77.1/69.6	60.4/63.6	77.4/69.9	72.8/68.0	77.9/70.8	72.8/71.9	53.7

polyprotein and 72.8% and 71.9% for aa and nt for the CP region, respectively. At the aa sequence in the NIb region, the PV-YO shared 80.5% identity with SMV (S42280), but they shared only 64.7% and 64.6% for the overall genome and polyprotein sequence, respectively (Table 6).

The nucleotide and amino acid sequence identities between PV-YO and other potyviruses were 43.0–61.5% and 24.4–57.0% for P1, 67.1–69.6% and 71.5–77.7% for HC-Pro, 57.0–60.9% and 42.9–47.6% for P3, 63.7–72.3% and 61.5–73.1% for 6KI, 67.3–71.0% and 74.8–77.8% for CI, 62.1–68.5% and 58.5–64.2% for 6K2, 40.0–69.5% and 72.1–79.3% for VPg, 42.0–69.6% and 70.4–74.5% for NIa, 68.2–72.9% and 72.9–80.5% for NIb, and 67.6–75.4% and 61.3–75.9% for CP (Table 6). In the 5’ NCR regions, the isolates shared 26.5–75.0% nucleotide identity. In the 3’ NCR regions, the isolates shared 47.2–59.4% nucleotide identity.

5.1.5. Phylogenetic analysis

Since no statistically significant recombination breakpoint occurrences in the polyprotein coding region of the isolates and other potyviruses were detected by the algorithms in RDP4, we then used a phylogenetic analysis to determine the genetic relationship among the PV isolates and other potyviruses using the neighbor joining and the maximum likelihood in MEGA software. The results revealed that the PV-AK, PV-OK, PV-YO, and PV-SA isolates from Akita, Okinawa, Yoron and Satsuma grouped in the same cluster, in a BCMV subgroup, meaning that the four isolates are identical. The bootstrap value also affirmed that the isolates are not closely related to PWV and EAPV-AO or IB strains found in Japan (Fig. 7, 8).

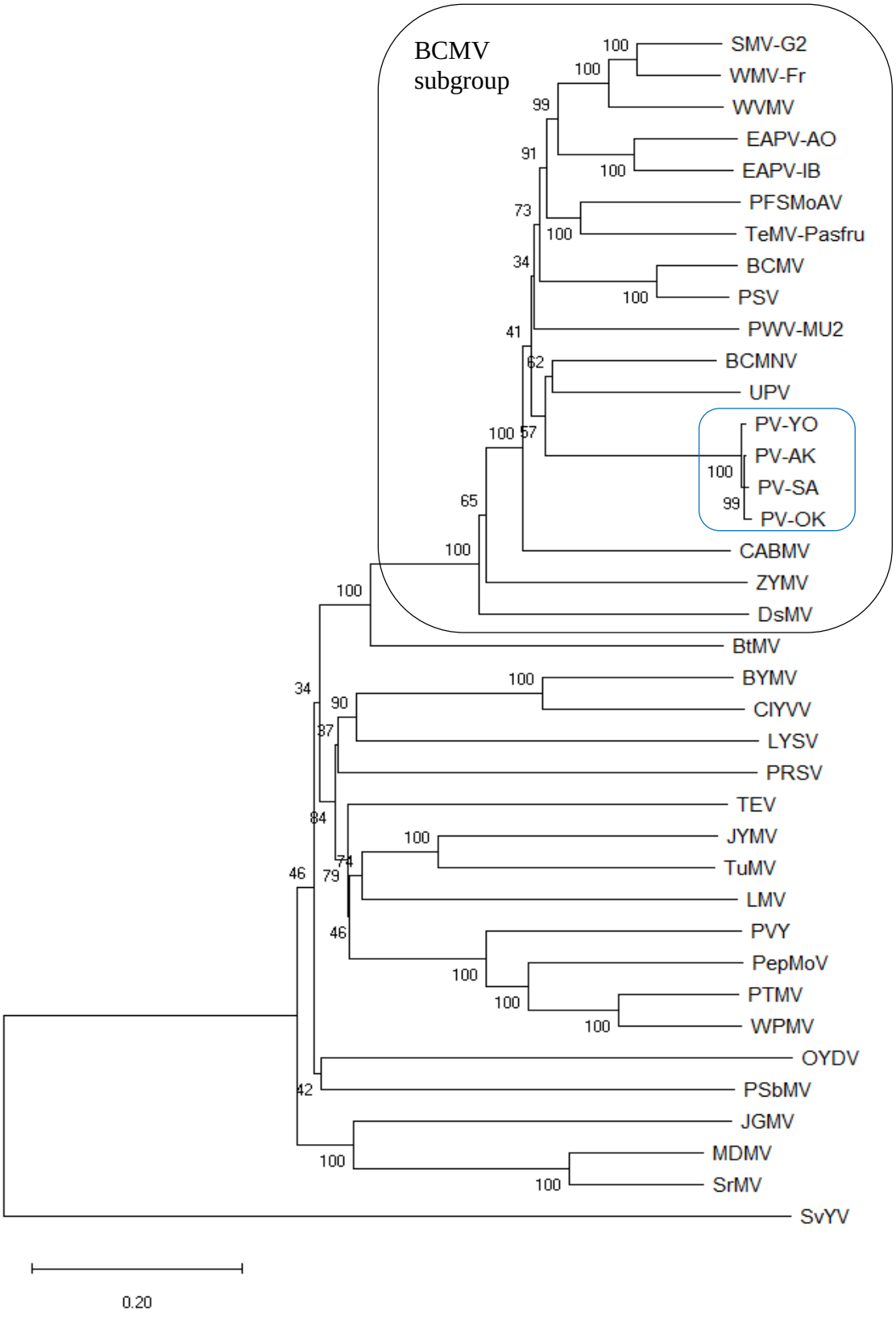

BCMV
subgroup
SMV-G2
WMV-Fr
WVMV
EAPV-AO
EAPV-IB
PFSMoAV
TeMV-Pasfru
BCMV
PSV
PWV-MU2
BCMNV
UPV
PV-YO
PV-AK
PV-SA
PV-OK
CABMV
ZYMV
DsMV
BtMV
BYMV
ClYVV
LYSV
PRSV
TEV
JYMV
TuMV
LMV
PVY
PepMoV
PTMV
WPMV
OYDV
PSbMV
JGMV
MDMV
SrMV
SvYV
0.20

Figure 7. Neighbor-joining tree made using the polyprotein sequences of the PV isolates and other 33 representative *Potyvirus* from GeneBank database. Percentage of in which the isolates clustered together was measured by 1000 bootstrap replications. The alignment of the isolates and tree analysis were generated by using MEGA X program. Accession numbers of the sequences used for constructing this tree are BCMNV (U19287), BCMV (AJ312437), BtMV (AY206394), BYMV (AY192568), CABMV (AF348210), ClYVV (AB011819), DsMV (AJ298033), EAPV-AO (AB246773), EAPV-IB (AB604610), JGMV (Z26920), JYMV (KJ789140), LYSV (AJ307057), LMV (P31999), MDMV (AJ001691), OYDV (AJ510223), PSV (U34972), PepMov (M96425), PTMV (AJ516010), PSbMV (D10930), PFSMoAV (MK449340), PRSV (X67673), PVY (X12456), PWV (HQ122652), SMV (S42280), SrMV (U57358), TEV (M15239), TuMV (AF169561), TeMV-PasFru (MG944249), WMV (AY437609), WPMV (AJ437279), WVMV (AY656816), UPV (MK110656), ZYMV (L31350) and SvYV as an outgroup (ALN38790).

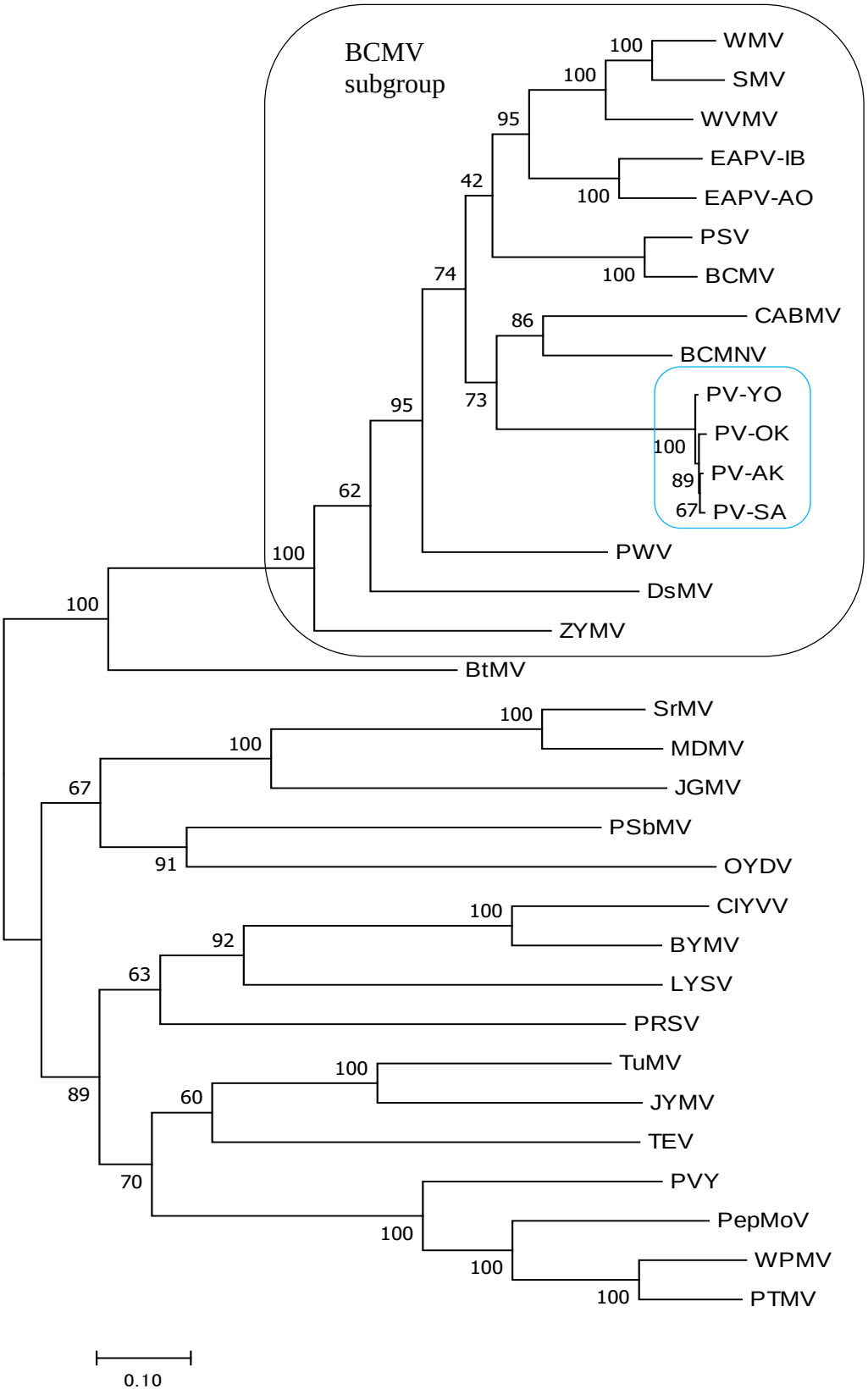

BCMV
subgroup
WMV
SMV
WVMV
EAPV-IB
EAPV-AO
PSV
BCMV
CABMV
BCMNV
PV-YO
PV-OK
PV-AK
PV-SA
PWV
DsMV
ZYMV
BtMV
SrMV
MDMV
JGMV
PSbMV
OYDV
ClYVV
BYMV
LYSV
PRSV
TuMV
JYMV
TEV
PVY
PepMoV
WPMV
PTMV
100
100
95
42
100
100
74
86
73
95
100
89
67
62
100
100
100
67
91
100
92
63
89
60
100
70
100
100
100
0.10

Figure 8. Maximum likelihood made using the polyprotein sequences of the PV isolates and other 29 representative *Potyvirus* from GeneBank database. Percentage of in which the isolates clustered together was measured by 1000 bootstrap replications. The alignment of the isolates and tree analysis were generated by using MEGA X program. Accession numbers of the sequences used for constructing this tree are BCMNV (U19287), BCMV (AJ312437), BtMV (AY206394), BYMV (AY192568), CABMV (AF348210), ClYVV (AB011819), DsMV (AJ298033), EAPV-AO (AB246773), EAPV-IB (AB604610), JGMV (Z26920), JYMV (KJ789140), LYSV (AJ307057), MDMV (AJ001691), OYDV (AJ510223), PSV (U34972), PepMov (M96425), PTMV (AJ516010), PSbMV (D10930), PRSV (X67673), PVY (X12456), PWV (HQ122652), SMV (S42280), SrMV (U57358), TEV (M15239), TuMV (AF169561), WMV (DQ399708), WPMV (AJ437279), WVMV (AY656816), and ZYMV (L31350).

5.1.6. Discussion

The virus etiology on passionfruit plants from four areas in Japan was examined. It was tentatively named the isolates PV-AK, PV-OK, PV-YO, and PV-SA, and all four isolates caused leaf mosaic, leaf curl, and fruit malformation. The biological, morphological, and molecular properties of the four PV isolates were utilized to identify them.

In general, the plants varied in their reactions to the four PV isolates and EAPV-AO. The four PV isolates induced a symptomless infection of *N. benthamiana*, which has been reported to be a potential host for many viruses (Goodin et al. 2008; Monjero et al. 2015; Van Dijk et al. 1987). In contrast, the plants inoculated with the EAPV-AO occasionally showed symptoms. The French bean cultivars reacted variously to the four PV isolates and EAPV-AO with reactions ranging from local lesions to systemic symptoms. Wylie and Jones (2011) and Chiemsombat et al. (2014) stated that French bean cultivars are known as indicator plants for potyvirus infection. Therefore, it was particularly interesting to see the differing reactions when French bean plants were inoculated with the PV isolates and EAPV-AO.

The inoculation with PV isolates led to systemic symptoms in cultivars native to Japan (Kairyo-Ohtebou and Hon-kintoki), whereas the EAPV-AO inoculation led to similar symptoms in cultivars from South America (Rico-23 and Rosinha). However, the reactions of these plants have still not been elucidated sufficiently to identify the individual viruses because environmental factors such as light, temperature and elevation of CO_2 levels may generate inconsistent systemic symptoms in the diseased plants (Aguilar et al. 2015; Blystad et al. 2015; Hamilton et al. 1981).

In contrast, the reactions of the cowpea cv. Kurodane sanjaku against the PV isolates differed greatly from those against the EAPV-AO, since this plant is immune to EAPV-AO but is systemically attacked by PV isolates. Thus, the different reactions of Kurodane sanjaku may be used for the early detection of both types of virus in Japan and would be of great benefit because the host range test is less expensive compared to ELISA and PCR-based tests.

The virus particles were flexuous and filamentous shape with an average length of 810 nm, which was typical of *Potyviridae* (Wylie et al. 2017). Since the alignment of the CP-coding region and the complete genome sequence identities among the four PV isolates were in the range of 98.6–99.6% and 98.3–98.5%, respectively, the PV isolates can be the same virus species (Adams et al. 2005b). The CP amino acid sequences of PV-AK differed slightly than those of PV-OK, PV-YO or PV-SA. Such differences are frequently found in potyvirus members due to heterogeneity at the level of nucleotide sequences (Shukla and Ward 1989; Wang et al. 2009a) or erroneous replication and recombination among plant RNA viruses (Malpica et al. 2002). The replication and recombination are natural processes in the life cycle of a virus that help the virus adapt to environmental conditions and may lead to the emergence of a new virus species. In addition, Kidanemariam et al. (2016) found that significant amino acid differences in the CP alignment of zantedeschia mild mosaic virus (ZMMV) strains from Taiwan and Australia were due to differences in geographic origin.

This analysis of the complete genome of the PV isolates using the EMBOSS procedure revealed that PV-OK shares 68.1% nucleotide identity with WMV, which is followed by BCMNV with which PV-OK shares only 65.6% and 66.6% identity of its overall genome and polyprotein-coding regions, respectively. Hence, following the recent

ICTV criteria, these four PV isolates were declared belong to a newly recognized member of the *Potyvirus* genus, since Adams et al. (2005b) and Wylie et al. (2017) explained that the demarcation line for distinct genus of *Potyviridae* is when a virus has ORFs at nucleotide identity < 46% and that the distinct species in the *Potyvirus* genus is when the complete genome, CP nucleotide, CP amino acid and 3′ NCR nucleotide identities of a suspected virus are < 85%, 76%, 80%, and 75%, respectively.

When the putative proteins of the four PV isolates were compared against several potyviruses, the P1 amino acid sequences of the PV isolates were the most divergent region, similar to the high rates of polymorphism found in the P1 protein of potyvirus members such as zucchini yellow mosaic virus and PVY (Urcuqui-Inchima et al. 2001). The NIb amino acid is the opposite, in that it is more homogenous, followed by VPg, CI and HC-Pro, as found in other potyviruses (Lan et al. 2015; Shukla et al. 1991). The results also demonstrated that the coding regions of the four PV isolates were more conserved compared to those of the NCRs, and the 3' NCR was less homogenous than the 5' NCR. This finding is slightly different from the case of the 3' NCRs of other potyviruses generally; because of those species' high similarity, they are commonly used to demarcate a species (Adams et al. 2005b).

The motifs are identical among the four isolates, but there are slight differences with respect to the conserved motif of potyvirus. As described in the "Results" section, one or two amino acids of the motifs in the HC-Pro and CP coding regions undergo substitution, and most of these motifs are associated with aphid transmission regulation (Babu and Hegde 2014; Blanc et al. 1997; Domier et al. 2003; Gal-On 2000; Huet et al. 1994; Li et al. 2014). In the present analysis, the spread of PV isolates in three distant locations in Japan (the main island of Okinawa and Yoron Island, the Kagoshima

mainland, and Akita Prefecture) may be attributable mainly to the movement of cloned plants rather than aphid transmission. Because cloned plants are produced and distributed over a short period, the infected virus has less opportunity to be transmitted by insects, and the genomic motif related to insect transmission may have been culled and lost.

This virus was initially found in Akita City by chance, but based on the fact that genomes of these isolates, which spread over a wide geographical region in a short time, were very similar, including their loss of an aphid-transmission gene, this virus species is thought to have spread by plants that were transported in the areas. And the gene for aphid transmission, which would have derived primarily from an ancestor of the four PV isolates, would have been lost in the process by which passionfruit cuttings were propagated somewhere in southern Kyushu. To ascertain whether those motifs in PV isolates are related to aphid transmissibility, study on aphid transmission of PV isolates is necessary.

The results of the phylogenetic tree based on the polyprotein, 5'- and 3'-NCR sequences showed that the PV isolates had high genetic similarities. The four PV isolates are not closely related biologically or phylogenetically to EAPV-AO, which nonetheless induces the same type of fruit malformation in several regions in Japan. The isolates are closely related with Ugandan Passiflora virus (UPV) and BCMNV. This analysis also strongly suggested that the nucleotide identities shared by the four PV isolates from three different geographical areas in Japan meet the current classification criterion as a distinct species in the *Potyvirus* genus. Taken together, the results lead to propose that this virus is a distinct passionfruit-infecting virus, appropriately named "East Asian Passiflora distortion virus (EAPDV)". Therefore, the four virus isolates used in this experiment have been designated as EAPDV-AK, -OK, -YO, and -SA.

5.2. Dual infection assay of EAPV and EAPDV in *Passiflora foetida*

5.2.1. Occurrence of coinfection of EAPV and EAPDV in passionfruit plant

Twenty-one passionfruit samples showing mosaic and leaf mottling/curly symptoms were collected. Prior to its RT-PCR amplification, the saps of passionfruit samples were inoculated mechanically to *P. foetida* and maintained until the leaves showed symptoms. EAPV and EAPDV are passionfruit disease-causing agents in Japan, so the primers used in amplification are aiming the CP coding region of EAPV and EAPDV. As a result, of the samples, 3 samples from Yuwan of Amami Oshima (2018), were infected only with EAPV. On the other hand, 4 samples in Nago City (2014), 6 samples from Ishigaki Island (2017), and 1 sample from Taketomi Island (2019) were infected with EAPDV, of which 4 samples from Ishigaki Island were also co-infected with EAPV. Other remaining samples from Nago City (2018) were uninfected, at least for EAPDV and EAPV where two distinct CP fragments were exposed in gel electrophoresis and it was validated the virus's identity as EAPV and EAPDV (Fig. 9). There is an obstacle to do artificial inoculation to passionfruit plant, So the dual interaction of EAPV and EAPDV in *P foetida* has been investigated.

5.2.2. Symptom expression in single infection of EAPV or EAPDV

In the initial phase of EAPV or EAPDV infection in *P. foetida*, disease symptoms progressed similarly. Both viruses produced mottling on the non-inoculated upper leaves at 3 dai. However, the degree of severity induced by EAPV increased more rapidly than induced by EAPDV. By 7 dai, the severity index in the plants inoculated with EAPV reached twice that of EAPDV-induced symptoms. The symptoms severity of the plants

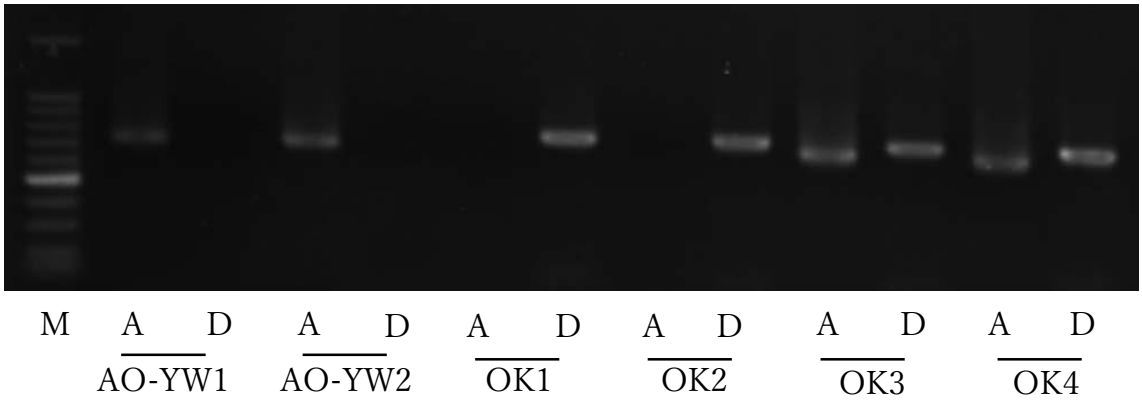


Figure 9. Detection of single and dual infection of EAPV and EAPDV in *P. edulis*represented by two isolates of Amami Oshima and four isolates from Okinawa prefecture using Reverse transcription-polymerase chain reaction (RT-PCR). Presented are images of 1% agarose gels following electrophoresis of RT-PCR product of EAPV (159 bp) and EAPDV (213 bp) targeting CP coding regions. M; DNA ladder (100 bp), A; Primer sets for EAPV, D; Primer sets for EAPDV, AO-YW1 and AO-YW2 were isolated from Yuwan, Amami Oshima in 2018, OK1 was isolated from Taketomi island, Okinawa in 2019, and OK2, 3 and 4 were isolated from Ishigaki Island in 2017.

infected with EAPV reached plateau by 7 dai, when the severity index was 2.05 to 2.32, whereas the degree of severity in the plants infected by EAPDV increased slightly at 21 dai, and reaching 1.79 at 28 dai (Fig. 10, 11).

5.2.3. Symptom expression in mixed and two-step EAPV and EAPDV infections

In the inoculation assay using *P. foetida*, coinfection with EAPV and EAPDV resulted in either synergistic interaction or slight delays in the appearance of symptoms. Mixed inoculation (A+D) and EAPV followed by EAPDV inoculation (A→D) elevated the degree of severity to 1.72-fold and 1.57-fold, respectively, higher than in the single inoculation with EAPDV at 28 dai (Figs. 10, 11). In contrast, two-step EAPDV followed by EAPV inoculation (D→A) induced milder symptoms over time compared to that induced by the A+D or by A→D.

5.2.4. Accumulation of EAPV and EAPDV under single and mixed infection in *P. foetida* plants

Virus accumulation was investigated using RT-qPCR at different time points: 1, 7, 14, and 28 dai (Figure 12). The number of RNA copies was represented as one virus particle, because each virus is encapsulated by a single strand RNA molecule. Therefore, we amplified the CP coding regions of EAPV and EAPDV to estimate each virus's titers. The accumulation value was normalized by *GAPDH* gene, and the average values were compared across three replications and two to three repetitions per unit in qPCR analysis. The value of normalized virus titer was presented in log 10 transformation. At the time of inoculation, both viruses, in either single, mixed, or two-step infections, were prepared under 0.88-0.99 x 10^6 copies/10 ml of initial inoculum concentration.

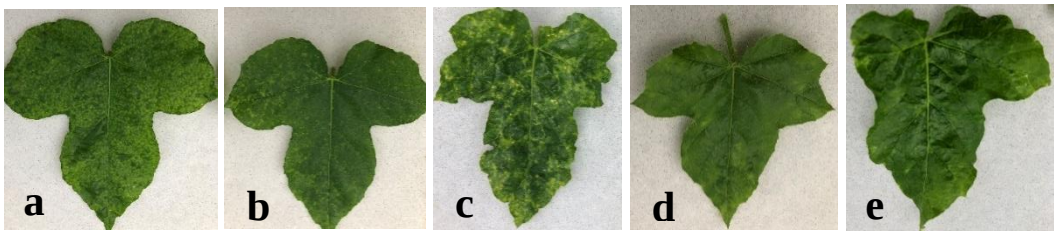


Figure 10. Systemic symptoms with mosaic and leaf mottling induced by single, mixed, and two-step infections with EAPV (A) and EAPDV (D). The observations were made at 28 dai. a A, b D, c A+D, d D→A, e A→D.

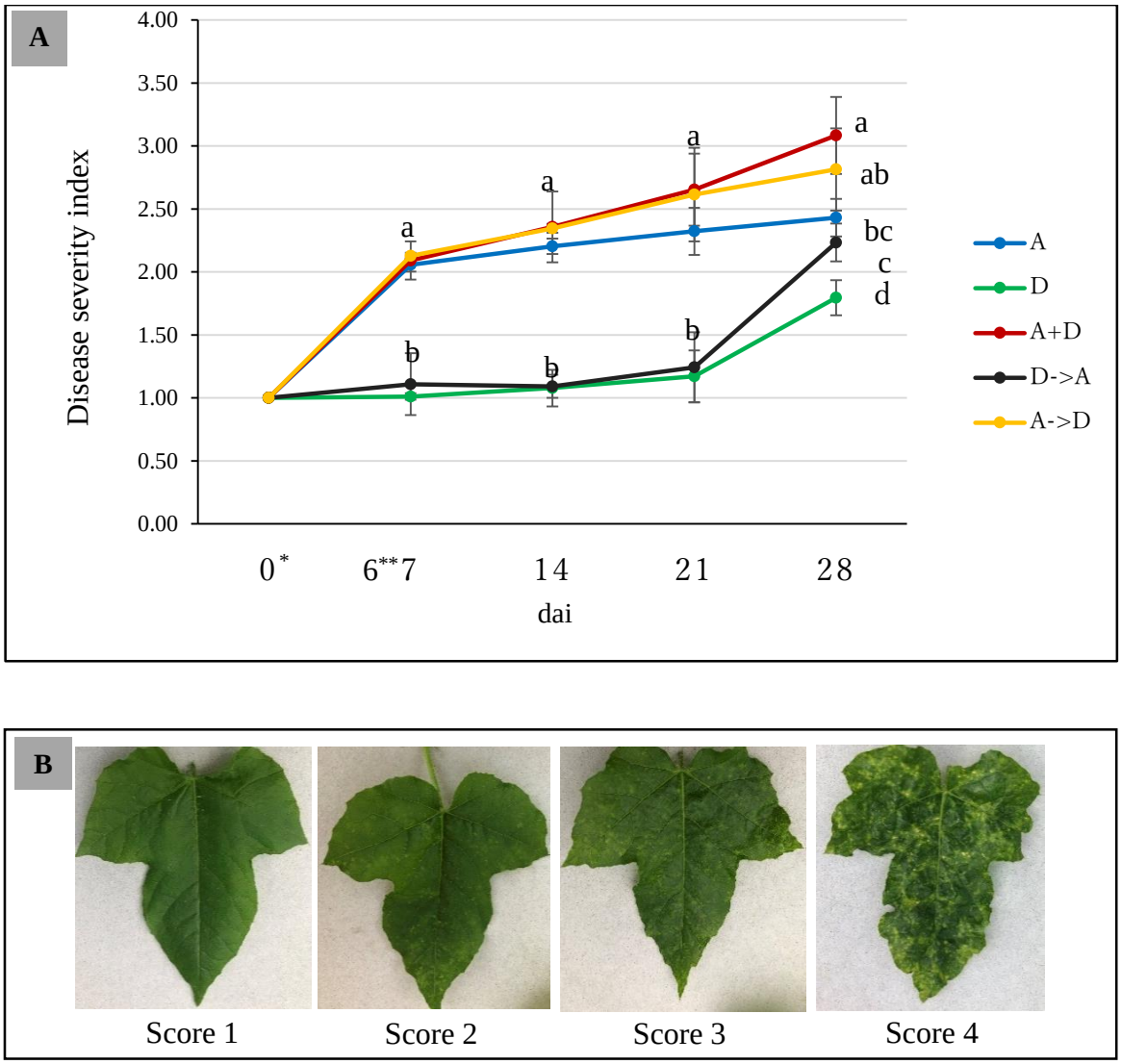


Figure 11. Symptom severity by time course of *P. foetida* infected with EAPV (A) and EAPDV (D) in single, mixed, and two-step infections as calculated using the formula $\delta t = \frac{\sum_{j}^{N}\left(\sum_{i}^{\lambda}\delta i\right)}{N}$, where δi is the damage on leaf i of plant j, λ is the number of leaves of plant j, N is the number of plants in a treatment, and t is dai. **A**; The bar of each time course represents the standard deviation of the mean. In statistical analysis, different letters shown at the same time course differ significantly according to Tukey's HSD test ($p<0.05$). *= first inoculation, **= second inoculation. **B**; Visual scale of symptom severity in *P. foetida* leaves. Score 1 = symptomless, 2 = slight mosaic, 3 = mosaic and mottling, 4 = severe mosaic and mottling.

As shown in Fig. 12A1 and -A2, at 1 dai, the EAPV titers in single and the mixed inoculations accumulated to higher levels than the EAPDV titers. Log 10 of EAPV titers reached 2.59 and 2.66, while those of EAPDV titers were only 1.32 and 1.13 in single and mixed inoculations, respectively, and differed significantly in statistical analyses. This accumulation increased in both treatments at 7 dai and differed significantly with the titer at 1 dai. EAPV titers decreased at 14 dai and remained low until 28 dai, and the accumulations did not differ significantly between single and mixed EAPV infections. In contrast, EAPDV titers increased from 1 to 14 dai in both single and mixed infections, where the log 10 of titers was 3.36 to 3.66, respectively. The increase of EAPDV titers continued in mixed infections, but the titers decreased slightly in the single inoculation at 28 dai (2.63). Although the EAPDV titers decreased in single infection at 28 dai, this accumulation still differed significantly with the EAPV titers in single inoculatin of EAPV and in the mixed infection.

5.2.5. Accumulation level of EAPV and EAPDV in two-step infections of *P. foetida*

The EAPDV titers in plants inoculated with D→A, and those inoculated with A→D at 1 dai or 7 days after the first inoculation were lower than, and significantly different from the EAPV titers (Fig. 12B1 and -B2). At 1 dai, the EAPDV titers in this order of inoculation were relatively the same as the titers in plants inoculated with a single inoculation or mixed infcections, where the log 10 virus titer were range from 1.00 to 1.67. From 1 to 14 dai or 7 to 28 dai of the first virus, the log 10 of the EAPDV titers in the D→A and the A→D increased to 2.28- and 4.83-fold, respectively. At 14 dai or 7 dai of the second virus, the titer of EAPDV in inoculation A→D was not different from that

at 1 dai, wherease that in D→A increased. The pattern of increased of EAPDV accumulation in D→A is parallel to the pattern in the single inoculation of EAPDV. At 28 dai or 35 dai of the first virus (EAPV), the titers of EAPDV in plants infected with D→A or in plant infected with A→D still increased. If EAPV titers was compared with EAPDV titers in the A→D and the D→A at 28 dai or 35 dai of the first virus, the EAPDV titers were higher and differed significantly from the EAPV titers in the statistical analyses.

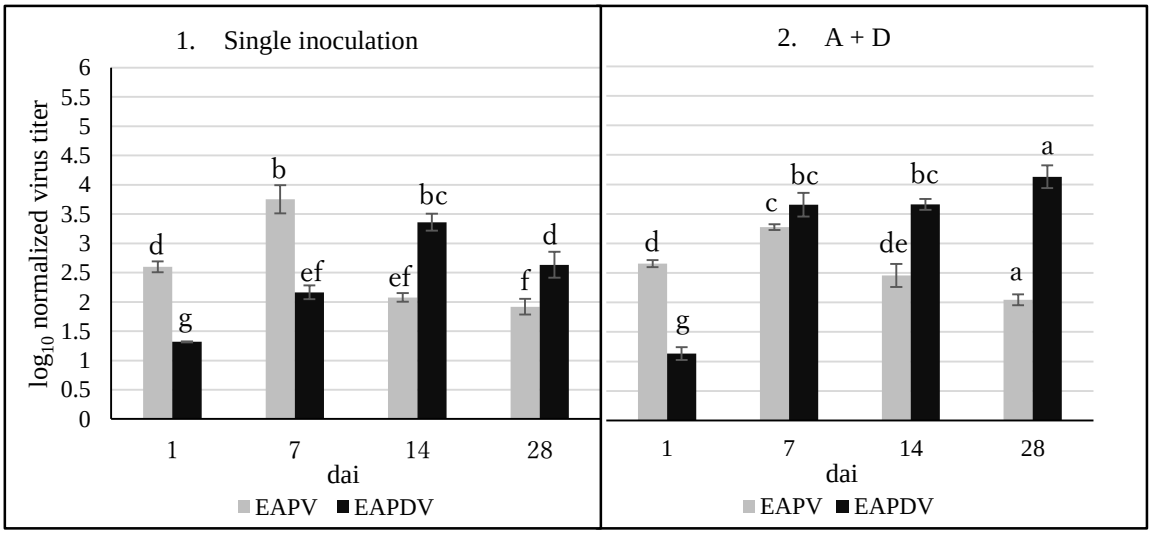
Significant differences in the temporal change in the virus amount were observed between EAPV and EAPDV. In the case of EAPV titers, whether EAPDV was inoculated first or second, the log 10 values of EAPV titers were between 2.07 and 2.53 and were not significantly different between A→D and D→A during in all detection periods in both inoculated and newly developed leaves. On the other hand, the log 10 value of EAPDV fluctuated widely in accumulation due to the presence of EAPV, and temporal change differed highly depending on the order of inoculation.

In the case of D→A, a synergistic effect appeared 1 dai with EAPV (and at 7 dai of EAPDV), and the effect became stronger until the following weeks. In the case of A→D, at at least 8 dai with EAPDV (14 dai with EAPV), the amount of EAPDV was slightly suppressed. After 22 dai with EAPDV, the synergistic effect due to EAPV began to appear.

5.2.6. Effects of single, mixed and two-step infection on pathogenicity related (PR) gene expression

The accumulations of plant genes in single, mixed, and two-step inoculations with EAPV and EAPDV at 14 dai (Fig. 13) were assayed. It was expected that the

A. Accumulation of virus titer in single and EAPV+EAPDV inoculations



B. Accumulation of virus titer in EAPDV→EAPV and EAPV→EAPDV inoculations

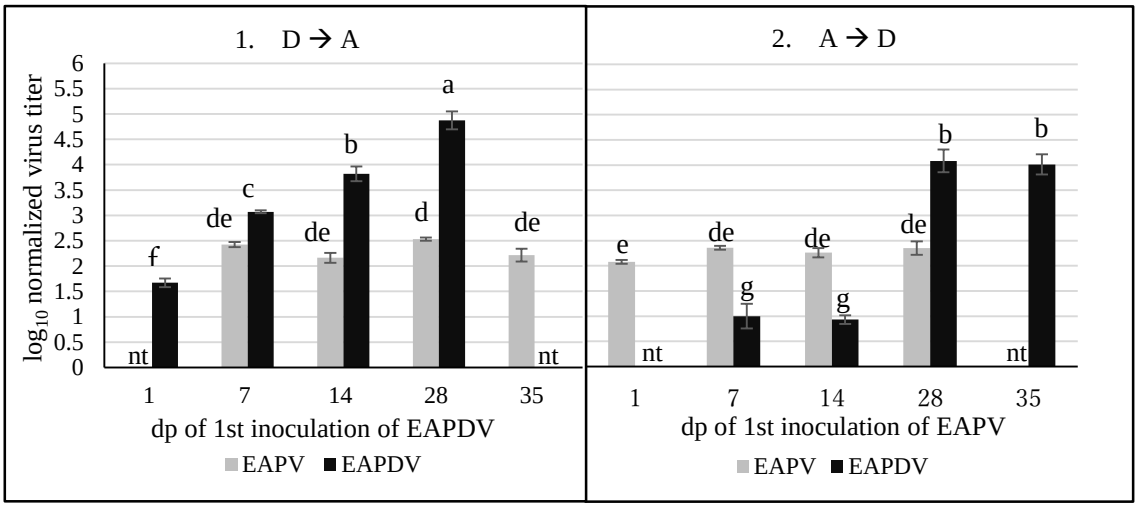


Figure 12. Quantitative RT-PCR showing titer levels of EAPV (A) and EAPDV (D) in *P. foetida* following single, mixed and two-step inoculations. In statistical analysis, different letters shown above the bar significantly according to Tukey's HSD test ($p<0.05$). A; In the single and mixed inoculations, the amounts of viral RNA in the inoculated leaves were examined 1day after inoculation (1 dai). From 7 dai, the amount of viral RNA presented in log 10 transformation from the fully expanded third leaves from the top were examined weekly until 28 dai for each virus. B; In the two-step inoculation, the amount of RNA of the first inoculated virus on the inoculated leaves was examined at 1 dai, and the inoculation with the second virus was performed on the fifth and sixth leaves from the top (identical to the leaves just above the first inoculated leaves) at 6 dai of the first virus. At 7 dai of the first virus (identical to 1 dai of the second virus), the amounts of the first viral RNA on the third leaves from the top and the second viral RNA in the inoculated leaves were examined. Finally, at 35 dai of the first (29 dai of the second virus), only the RNA of only second virus on the upper leaves was examined. The *GAPDH* gene *P. foetida* was used as an endogenous reference control. nt; not tested.

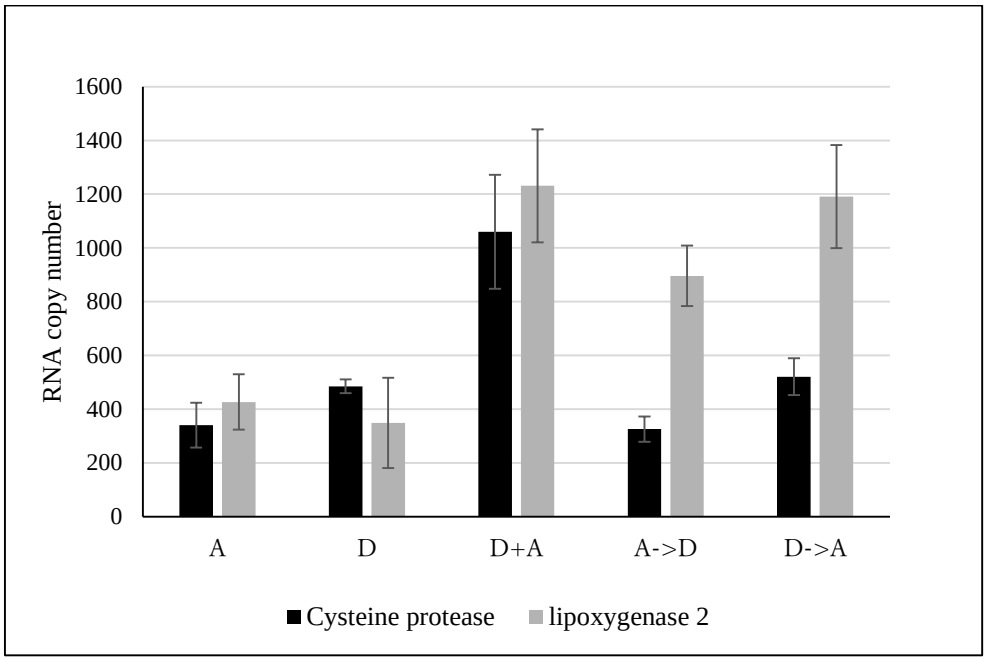


Figure 13. PR gene expression level in EAPV (A) and EAPDV (D) in single, mixed, and two-step infections at 14 dai. Data represent means ± standard deviations of five replicates.

accumulation levels would be already homogenous by 14 dai. To access reliable data, the accumulation value was normalized by *GAPDH* gene and the averaged values were compared across three replications. In general, the mixed and two-step inoculations led to more PR genes being differentially regulated in comparison to the single inoculation. Examination of the expression levels revealed that *LOX2* gene was up-regulated by the mixed-, and two-step inoculations with EAPV and EAPDV. The accumulation of genes in the mixed, and two-step inoculations were elevated with fold changes ≥ 3.52 in comparison to the single inoculation with EAPV or EAPDV.

The fold change of cathepsine B-like cysteine protease gene were significantly increased up to $\geq 2{,}8$ by mixed infections compared to single infections with either EAPV or EAPDV. However, the accumulation decreased with fold change $\geq 0{,}9$ in the two-step infections of EAPV followed by EAPDV and constantly with folds change $\geq 1{,}5$ in the two-step infection EAPDV followed by EAPV compared with single infections with either EAPV or EAPDV (Fig.13).

5.2.7. Discussion

PWD is an important disease in passionfruit because it damages fruit quality. PWD limited in one town and two villages of the southern part of Amami Oshima in 1992. By 1997, PWD and the pathogenic virus EAPV has spread throughout the island. And during following period, EAPV spread to Satsuma Peninsula and Osumi Peninsula on the mainland of Kagoshima Prefecture. Subsequently, passionfruits in Kagoshima Prefecture, including Amami Oshima, were thoroughly replaced with virus-fee healthy cuttings. Due to this effect, since 2010, the only places in Kagoshima Prefecture where EAPV remains

are limited to a few places in southern Amami Oshima (Iwai 2017). In this study, three EAPV isolates were found at Yuwan, Amami Oshima.

In 2014, PWD symptoms were also found in passionfruit in several locations of Okinawa. A molecular analysis using CP- and PP-coding regions showed that the recent isolates isolated from PWD-passionfruit samples was not caused by EAPV, but a new virus named EAPDV. These discoveries encourage a wider collection of diseases in the Okinawa region. It turns out, molecular analysis showed that EAPDV also spread in Taketomi and Ishigaki Islands. Prior to 2014, Ishigaki was the center of passionfruit vegetative seed suppliers for whole Japan. Thus, it is likely that EAPDV originated in this region spread to Okinawa, Kagoshima and Akita prefectures along with the distribution of plants. Subsequently, PWD was no longer detected in the Nago City, where the EAPDV was first discovered, by replacing with healthy cuttings. Four EAPV isolates from passionfruit in Ishigaki revealed that EAPV has spread to southern Japan (Fig.9). Even an interesting finding from this preliminary study is that the dual infection of EAPV and EAPDV. Therefore, a new experiment was performed to clarify the relationship between the two viruses.

The assay demonstrated that *P. foetida* can be affected by these two viruses simultaneously. Leggat and Teakle (1975) indicated that *P. foetida* is an alternative host and potential reservoir for PWV in Australia. In South-East Queensland, the plant is co-infected commonly by Passiflora virus Y (PaVY) and PWV (Parry et al. 2004).

Single and dual infection of EAPV and EAPDV induced similar types of symptoms, such as systemic mosaic and leaf mottling. In the case of single infection, the symptoms of EAPDV are milder than those of EAPV. However, the most interesting findings are that EAPV and EAPDV develop different symptomatic outcomes and that

the accumulation of RNA of each virus during dual infection depends on the inoculation order on *P. foetida*. The data showed that EAPV and EAPDV coinfection in *P. foetida* elevated symptom severity over that from single infection. Infections in the order of A→D showed synergism between the viruses. Synergism in this context is defined as the induction of more (or more severe) symptoms resulting from co-infection with two or more viruses than would result from the sum of symptoms occurring with single infections of the same viruses had the infection occurred separately.

Often, titers of one or both viruses also increase in co-infection (Syller 2012). It was suspected that EAPV appears to play a role in determining the symptoms, where infection with it followed by that with EAPDV causes more severe symptoms than infection with D→A. Synergistic interactions have been reported in the literature between two potyvirid species, wheat streak mosaic virus (WSMV) and triticum mosaic virus (TriMV) (Tatineni et al. 2010), and in co-infections between unrelated viruses such as PVY and PVX, or between papaya mosaic virus (PapMV) and papaya ringspot virus (PRSV) (Chaves-Calvillo et al. 2016; Nie et al. 2013; Liang et al. 2016). The interaction between unrelated viruses is more common in nature, and generally either virus plays a role in determining symptom severity (Liang et al. 2016; Vance et al. 1995).

In mixed infection with two potyviruses, WSMV and TriMV, the replication of both increased during synergistic interaction (Tatineni et al. 2010). In contrast, the data indicated that EADPV titers in mixed and two-step inoculations with EAPV and EAPDV are higher than in single infection, while the titer level of EAPV did not change. This result was the same as in the synergistic interactions between potyvirus and potexvirus (PVY and PVX in *N. benthamiana*), where only PVX titers increased while PVY did not (Liang et al. 2016; Vance 1991). Additionally, a study by Čeřovska et al. (2008) showed

the same phenomenon and found that the factor triggering the increase in PVX replication in synergistic interaction between PVX and PVY was HC-Pro of PVY, where HC-Pro suppresed PTGS expressed by the plants.

In infection with D→A, EAPDV titers increased sharply at 28 dai, which is 22 days after EAPV inoculation. Likewise, in A→D, the titer of the latter also increase at 22 dai. These data reinforce our conjecture that EAPV plays a role in EAPDV replication in mixed infection. The absence of a significant increase in EAPDV titers in mixed inoculation at 8 dai (14 dai for EAPV, on Fig. 12B2) might be attributable to concurrent competition in cells when exploring the sources (González-Jara et al. 2009).

Some studies have explained that, mainly, there is no correlation between the expression of symptoms in the host and the virus titers (González-Jara et al. 2004; Kokkinos and Clark 2006). The increase in PapMV titers in synergistic interaction with PRSV is not associated with severe symptom phenotype in the host (Cháves-Calvillo et al. 2016). The enhancement of a detrimental symptom in synergistic interaction of PVX and PVY is host-dependent and not attributable to the increase in PVX titers (Garcia-Marcos et al. 2009). The data also demonstrated that the titers of EAPV and EAPDV are not likely to be responsible for the enhanced symptom expression and synergism in single inoculation or D→A inoculation. In a single infection, EAPDV titer was higher than that of EAPV at 28 dai, but symptom expression was milder (Fig 10, 13d). Then, the titer of EAPDV was higher with D→A inoculation, but this condition did not induce more severe damage to its host than the mixed inoculation or that with A→D. As well, even though EAPV titers were at low levels in all treatments, EAPV still caused severe symptoms in single, mixed and order inoculation, A→D. In addition, I suspected that EAPDV titer

might play a role in the expression of symptom in mixed and A→D but we need to conduct a more detailed experiment in future to prove it.

When a plant was infected with a virus, various type of symptoms will emerge as plant defense responses. In this case, the plant will express several PR genes (García-Marcos et al. 2009). In the experiment, we selected two PR genes, *LOX2* and cathepsin B-like cysteine protease genes. *LOX2* gene, equal to *9*- and *13 LOX*, is involved in localized cell reaction as a hypersensitive responses (HR) (Nishiguchi et al. 2018). Several known PR genes expressed by *P. edulis* such as *LOX2* and cathepsin B-like cysteine protease genes, are involved in a hypersensitive reaction to bacterial infection (Munhoz et al. 2015). However, there is no report about PR genes expression during viruses infection in *P. edulis* or *P. foetida*. It was refered to Aquilar et al. (2015) and García-Marcos (2013), which found that synergistic PVY-PVX infection enhanced *LOX2* gene expression on *N. benthamiana*. In this study, *LOX2* gene expression was higher in dual infection than in single. However, the *LOX2* gene expression also increased in D→A, which induced milder systemic symptoms at 28 dai. This finding does not allow us to verify the relationship between *LOX2* gene expression directly and a plant's necrosis symptom. In addition, involvement of several other genes during the interaction and expression of several periods of time are necessary to be observed in order to know the gene's involvement in the interaction (Thomas and van der Hoorn 2018).

While the cathepsin B-like cysteine protease gene was referred to Hanssen et al. (2011) in which an aggressive strain of pepino mosaic virus isolate enhanced cathepsin B expression in tomato than did by the mild isolate even though the viral titers level were the same. Cathepsin B is a cysteine protease found in lysosomes in plant cells, where it mediates plant cell death (PCD) (Mort and Buttle 1997). Few reports provide evidence of

cathepsin B regulation in HR-induced-PCD in plants. However, recently, it was found that cathepsin B in tomato was induced to a higher level by the aggressive isolate of pepino mosaic virus than by the mild isolate even though the viral titers were similar (Hanssen et al. 2011). The present study showed, that cathepsin B-like cysteine protease gene accumulated to the highest levels in mixed inoculation, lower levels by EAPDV single inoculation, and to the lowest levels in D→A. In contrast to the mixed inoculation, which induced severe symptoms, EAPDV single inoculation and A→D both induced mild *P. foetida,* symptoms. The accumulation was low in the single EAPV inoculation and in the inoculation with EAPV followed by EAPDV, both of which caused severe symptoms. Therefore, we assumed that the requirement of the protease might be clear in these plant-pathogen interactions because the treatments induce different accumulations of the protease. It was predicted that several other factors are involved in the interaction due to the inconsistency of protease accumulation. Additionally, a mechanism was reported by which several proteases link to a defense whose role in immunity is still completely unknown (Ilyas et al. 2015; Thomas and van der Hoorn 2018).

From the expression of these two PR genes, it was determined that *LOX2* and cathepsin B-like cysteine protease expression are directly related to plant's necrosis systemn or instead are not involved in a resistance program or hypersensitive reaction. In addition, the involvement of several other genes during the interaction and expression of genes over several periods of time must be observed in order to know each gene's involvement in the interaction (Thomas and van der Hoorn 2018).

The data of virus accumulation and symptom appearance suggested that EAPDV can exploit *P. foetida* as a reservoir plant. The presence of EAPV after infection with EAPDV is also beneficial for both viruses. The symptoms caused by this interaction in *P.*

foetida are milder despite of the high titer accumulation. Therefore, the interaction can prolong the existence of both viruses in nature. The high accumulation of EAPDV in *P. foetida* can also increase the efficiency with which insects spread the virus. Even there is no occurrence of EAPV and/or EAPDV from naturalized in *P. foetida* in Okinawa or in worldwide, the possibility of this plant to be a potential intermediate host of EAPV or EAPDV in the future is very high. This plants is infected easily by several viruses using a mechanical inoculation method.In a greenhouse study, EAPV or EAPDV propagated well in this plant under different condition (Riska et al. 2019). The factors that inhibit EAPV/EAPDV natural infections in *P. foetida* in Okinawa are still not clear. The reason why the natural infection did not occur is that recently the passionfruit and *P. foetida* habitats on Okinawa Islands have been separated. If the passionfruit field approaches the habitat of *P. foetida*, the infectiouse virus may be transferred, and the wild species may serve as an intermediate host. As reported by Yonaha et al. (1979) that around 40 years ago, *P .foetida* in Okinawa was found to be infected by several viruses, where aphids, *Myzus persicae* as an agent transmitted some viruses to *P. foetida* which spread around passionfruit fields on Okinawa Island. In addition, as mentioned in the introduction that in other countries this plant is the host of several viruses including potyvirus, PVY and PWV.

VI. GENERAL DISCUSSION AND CONCLUSION

Potyvirus is one of the genera in plant viruses causing great damage to agricultural crops worldwide. This genus is also reported to be a major cause of yield loss in passionfruit plants and the major known disease is passionfruit woodiness disease (PWD). In Australia, PWD is caused by PWV. PWD was first discovered in Japan in the Amami-Oshima area in 1986, but from the results of the study of Iwai et al. (2006) that virus was a new type of potyvirus, namely EAPV-AO (AB246773) whose protein homology only 65.4% with PWV (HQ122652). In 2013, a PWD-like symptoms were found again in passionfruit in Akita in 2013, then successively found in plantations in the Okinawa, Yoron, and Satsuma areas during 2014 to 2015.

In the present study, four isolates (PV isolates) showing a PWD-like disease obtained from the region in Akita, Okinawa, Yoron, and Satsuma Japan were examined. The sequence comparisons, phylogenetic analyses as well host range test indicated that the isolates were not related to EAPV found previously.

Different with EAPV, the four PV isolates induced a symptomless infection of *N. benthamiana*, systemic symptoms in cultivars of French bean native to Japan (Kairyo-Ohtebou and Hon-kintoki) and systemic necrosis to Cowpea cv. Kuradane sanjaku. Even the host range assay was stated a less effective method, based on the assay to both types of virus, those host plants could be used for early detection of both viruses and the research is become less expensive compared to ELISA and PCR-based tests.

The PV isolates are the same species due to the alignment of the CP-coding region and the complete genome sequence identities among the four PV isolates were in the range of 98.6–99.6% and 98.3–98.5%, respectively, and PV-OK as representative share only 65.6% and 66.6% identity of its overall genome and polyprotein-coding

regions, respectively with BCMNV. Therefore, these isolates could be grouped into genus of *Potyvirus* as their particle were flexuous and filamentous shape with an average length of 810 nm (Wylie et al. 2017). All four isolates genomic features are a typical of *Potyvirus* and belong to members of BCMV subgroup which includes several different viruses infecting both monocot and dicot, legume and non-legume plants (Berger et al. 1997). Since Adams et al. (2005b) and Wylie et al. (2017) explained that the demarcation line for distinct genus of *Potyviridae* is when a virus has ORFs at nucleotide identity < 46% and that the distinct species in the *Potyvirus* genus is when the complete genome, CP nucleotide, CP amino acid and 3′ NCR nucleotide identities of a suspected virus are < 85%, 76%, 80%, and 75%, respectively. While such differences between CP of PV-AK and others are due to heterogeneity at the level of nucleotide sequences (Shukla and Ward 1989; Wang et al. 2009a) or erroneous replication and recombination among plant RNA viruses (Malpica et al. 2002). The isolates are proposed as the novel species named as East Asian Passiflora distortion virus (EAPDV).

The motifs in HC-Pro and CP of the isolates are slightly differences with the conserved motif of potyvirus, where their functions are related to regulation of aphid transmission. It was assumed that the occurrence of mutation in nucleotide sequences of the isolates, changes their motifs and function, may be caused by the environmental effect. The virus-infected cloned plants produced and distributed over short period of time induced loss of their ability to be transmitted by aphid.

In the present study, a passionfruit sample from Ishigaki islands was affected by EAPV and EAPDV simultaneously. This is the first report of the mixed infection of both virus in Japan. The assay demonstrated in *P. foetida* showed that the plants can be affected by these two viruses simultaneously. Mixed infections are a common phenomenon in

plant viruses that occurs between potyvirus and non-potyvirus. In this study, EAPV and EAPDV accumulation in plants depends on the order of inoculation on *P. foetida*. Mixed infection of EAPV and EAPDV and the order infection of A→D elevated symptom severity over that from single infection. The most common interaction is synergistic interaction whereas one virus helps to increase accumulation other viruses. However, inoculation of EAPDV followed by EAPV caused milder symptom. This phenomenon is thought to be a form of adaptation by viruses. The virus will always try to prolong its life in a host and will cause problems that are not excessive to plants. Another possibility is that plants also always pass up defenses against virus.

The data of virus accumulation and symptom appearance indicated that titers of EAPDV in mixed and order inoculation of EAPV and EAPDV are higher than in single infection. Interaction between potyvirus and potexvirus (PVY and PVX in *N. benthamiana*) showed the same result where only PVX titer increase while PVY did not (Liang et al. 2016; Vance 1991). These data support the hypothesis that EAPV plays a role in EAPDV replication in mixed infection. However, EAPDV titers are low in mixed inoculation at 8 dai (14 dai for EAPV, on Fig. 12B2). This occasion might be caused by concurrent competition in cells when exploring the sources (González-Jara et al. 2009).

Usually the PR gene will react immediately after an infection. In this experiment, two PR genes, *LOX2* and cathepsin B-like cysteine protease were used due to both genes are involved in a hypersensitive reaction to bacterial infection (Munhoz et al. 2015). Because there is no report about PR genes expression during viruses infection in *P. edulis* or *P. foetida*, the gene were instead referred to Aquilar et al. (2015) and García-Marcos (2013), which found that synergistic PVY-PVX infection enhanced *LOX2* expression on *N. benthamiana*. *LOX2* expression was higher in dual infection than in single but also

increased in D→A, which induced milder systemic symptoms at 28 dai. The inconsistency of gene expression might be due to involvement of several other genes during the interaction (Thomas and van der Hoorn 2018). Thus expression of the gene are also affected by environment condition. So it is necessary to observe the gene expression during several periods of time.

The present study showed, as well as *LOX2*, it was found inconsistency of cathepsine B-like cysteine protease expression. The gene accumulated to the highest levels in mixed inoculation, lower levels by EAPDV single inoculation, and to the lowest levels in D→A. Subsequently, in mixed inoculation, EAPDV single inoculation and A→D both induced mild *P. foetida*, symptoms. The accumulation was low in the single EAPV inoculation and in the inoculation with EAPV followed by EAPDV, both of which caused severe symptoms. Therefore, the protease might be required in these plant-pathogen interactions but several other factors may involved in the interaction.

The data of virus accumulation and symptom appearance suggested that *P. foetida* is potential to be an intermediate host of EAPV and EAPDV. EAPDV with presence of EAPV caused milder despite of the high titer accumulation in plants. There is no report of EAPV and/or EAPDV infect *P. foetida* in Okinawa or in worldwide but this plants is infected easily by several viruses using a mechanical inoculation method. If the passionfruit field approaches the habitat of *P. foetida*, the disease incidence may occur and the virus are transferred and the wild species may serve as an intermediate host.

Conclusion

Currently, list of the quarantine plant virus infecting passionfruit in Japan is restricted to East Asian Passiflora virus. The increase number of potyviruses identified infecting passionfruit in Japan and evidence of the virus in Okinawa and Ishigaki in this study will provide valuable information to establish a quarantine plant virus list that useful for conducting plant disease control strategy relating to the movement of plant material around Japan area and exported from other area.

Subsequently, this thesis strived to obtain a deeper understanding of host pathogen interaction particularly synergistic interaction between EAPV and EAPDV in *P. foetida*, a better understanding in interaction of several pathogenicity-related genes in plant mixed infection and confirmation of *P. foetida* which present as an alternative reservoir plant for EAPV and EAPDV could help to ameliorate crop losses to virus diseases by providing improved phytosanitary methods, prevention and treatment of viral diseases.

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