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REVIEW

Major Citrus Viruses in Japan

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Abstract

Citrus species grown in Japan are affected by three major viruses: satsuma dwarf virus (SDV), citrus tatter leaf virus (CTLV, apple stem grooving virus), and citrus tristeza virus (CTV). SDV has spread to other countries, such as China, Korea, and Turkey, apparently through unchecked budwoods and nursery trees; it is natural to assume that some of these contaminated plants harbored CTLV and CTV as well. Thus, citrus viruses in Japan are not only a domestic problem, they are also transboundary threats to agricultural production. This article reviews the occurrence and history of SDV, CTLV, and CTV in Japan as well as recent research developments in Japan and other countries.

Discipline: Agricultural Environment
Additional key words: quarantine program, transboundary pest, virus elimination

Introduction

Graft-transmissible diseases have been a common problem affecting citrus production in Japan. The primary cause of these diseases is the improper vegetative propagation of contaminated budwoods of popular cultivars by tree growers and nursery workers. New cultivars developed by official breeding programs are usually released after eliminating graft-transmissible agents. However, nursery trees become infected with viruses and viroids during propagation and circulation. Satsuma dwarf virus (SDV) has spread to other countries, including China, Korea, and Turkey, apparently through the introduction of unchecked budwoods and nursery trees; it is natural to assume that some of these contaminated plants were mix-infected with citrus tatter leaf virus (CTLV; apple stem grooving virus, ASGV) and citrus tristeza virus (CTV) as well. Further, CTLV and some isolates of CTV had originated in China, and had introduced to Japan in old days before plant quarantine was established. Thus, some citrus viruses and viroids in Japan should be recognized internationally as major transboundary pests, and it would be useful to understand incidence trend, characters of citrus viruses, and viroids around Japan.

SDV, CTLV, CTV, citrus exocortis viroid (CEVd), and some citrus viroids (CVds) are widely distributed and seriously decrease citrus production in Japan. Other viruses and viroids, such as citrus yellow mottle virus and citrus vein enation virus, are endemic or cause relatively limited economic losses. With citrus viruses and viroids that had been originally reported in Japan having also been reported outside of Japan, a comprehensive review regarding these agents has not been published since the early 1980s (Miyakawa & Yamaguchi 1981), except for a mini review on SDV (Iwanami 2010). Additionally, classical Japanese articles on citrus viruses and viroids that had been unavailable for most of the foreign readers are currently easily found online using search engines (e.g., Google Scholar) and may be translated into any language. Therefore, it is time to introduce such classical Japanese papers that had been rarely recognized among citrus virologists abroad. In this context, I herein provide a general overview of the occurrence and history of the three major viruses (SDV, CTLV, CTV) in Japan as well as recent advances in related research in Japan and in East Asia (China, Taiwan, and Korea). A review on other citrus viruses and viroids in Japan will be published elsewhere.

SDV

1. Occurrence

(1) Occurrence in Japan

SDV is widely distributed in major citrus-producing areas in Japan. Previous research revealed that SDV

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infects most citrus cultivars and related species (Iwanami et al. 1993b, Miyakawa 1969). Infected satsuma mandarin [*Citrus unshiu* (Swingle) Marcow.] trees typically develop relatively small and/or boat- or spoon-shaped leaves with short internodes, resulting in a stunted appearance. In contrast, SDV infections of orange [*Citrus* × *sinensis* (L.) Osbeck], tangor, a hybrid between tangerine [*Citrus reticulata* Blanco] and orange, and other hybrid cultivars result in leaves that are smaller and narrower than normal (but not boat- or spoon-shaped) as well as decreased tree vigor (Fig. 1A). In most cases, SDV infections lead to severe decreases in fruit quality as well as yield losses (Imada et al. 1980).

(2) Occurrence outside of Japan

The first reported SDV infection outside of Japan occurred in Turkey (Azeri 1973). In 1998, SDV infections of satsuma mandarin were at an amount of 2% and 31.6% in the Aegean region and in the Mediterranean region, respectively (Uygun & Satar 2008). In East Asia, SDV has been detected in China and Korea (Zhou 2018, Hyun et al. 2017). However, SDV has apparently been controlled in China since 2001 through the implementation of a virus-free scheme (Zhou 2018). In Korea, SDV commonly infects satsuma mandarin as well as medium- to late-maturing hybrid cultivars (e.g., "Setoka" and "Shiranui") on Jeju Island (Hyun et al. 2017). To date, there have been no reports of the occurrence of SDV in other countries.

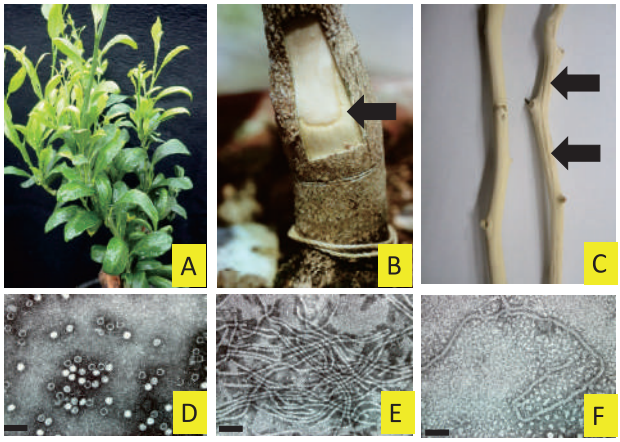

Fig. 1. Symptoms and virions of satsuma dwarf virus (SDV), citrus tatter leaf virus (CTLV), and citrus tristeza virus (CTV)

A, Boat-shaped symptoms on leaves of satsuma mandarin infected with SDV; B, a bud-union crease (arrow) appeared on satsuma mandarin on trifoliate rootstock infected with CTLV (photo by Meisaku Koizumi); C, pitting (arrows) appeared on a yuzu twig infected with CTV; D, E and F, virions of SDV, CTLV, CTV, respectively with bars indicating ca. 100 nm.

2. Biological and molecular biological properties

With a previous study demonstrating that SDV can naturally infect woody plant species growing near infested citrus fields, including *Daphniphyllum teijsmannii* Zoll. ex Kurz. and *Viburnum odoratissimum* Ker-Gaul. var. *awabuki* (K. Koch) Zabel (Nakazono-Nagaoka et al. 2014), it is seen that the symptoms in some medium- to late-maturing citrus cultivars, such as "Harumi," are relatively mild at least until four years after infection (Kato et al. 2019). Moreover, *V. odoratissimum* windbreaks can promote SDV infestations of citrus fields (Mae 1984). Furthermore, in an earlier study, enzyme-linked immunosorbent assays (ELISAs) detected SDV infections of yew plum pine [*Podocarpus macrophyllus* (Thunb.) Sweet], East Asian eurya (*Eurya japonica* Thunb.) Japanese cypress [*Chamaecyparis obtusa* (Sieb. et Zucc.) Endl.], and tea [*Camellia sinensis* (L.) Kuntze] (Mae 1984). However, ELISAs can produce false positives; therefore, the results needed to be re-examined using other detection techniques like RT-PCR. Other studies determined that SDV can be readily transmitted to many herbaceous species, including sesame (*Sesamum indicum* L.) and *Physalis floridana* Rydb., and then back to citrus plants via mechanical inoculation (Iwanami 2010, Iwanami et al. 1991a). Published field trial results strongly suggest SDV is a soil-borne virus (Isoda & Gyoutoku 1990). During a 31-year survey, SDV was observed to move approximately 0.8 m per year on average (Katagi & Ushiyama 1990), but the vector remains unknown (Iwanami 2010).

Polyhedral SDV virions are non-enveloped and approximately 26 nm in diameter (Fig. 1D). These virions have two distinct coat proteins with a molecular weight of approximately 42,000 and 22,000. Bipartite genomic RNA sequences (RNA1 and RNA2) are single-stranded (+)-sense RNA molecules that are encapsidated in separate virions. Both RNA sequences, which are polyadenylated at the 3′ terminal, encode a single polyprotein (Iwanami 2010). Comoviruses and nepoviruses have a similar virion morphology and genome organization, suggesting that viral genome-linked proteins are likely attached to the 5′ terminal of SDV RNA sequences, but this will need to be experimentally confirmed.

3. Taxonomy and strains

(1) Taxonomy

SDV is classified to the virus species of *Satsuma dwarf virus*, which is the type species of the genus *Sadwavirus*. The exemplar isolate (S-58) has been used extensively for serological and molecular characterizations in Japan (Iwanami 2010). The reorganization of the genus *Sadwavirus* to create three new subgenera was recently

proposed (Sanfaçon et al. 2020). According to this reorganization, SDV belongs to the subgenus *Satsumavirus* in the genus *Sadwavirus* of the family *Secoviridae* of the order *Picornavirales* (Table 1).

(2) Strains

Citrus mosaic virus (CiMV), navel orange infectious mottling virus (NIMV), Natsudaidai dwarf virus (NDV), and Hyuganatsu virus (HV) mainly affect mandarin, navel orange [*Citrus* × *sinensis* (L.) Osbeck], natsudaidai, (*Citrus natsudaidai* Hayata), and hyuganatsu (*Citrus tamurana* hort. ex Tanaka), respectively (Ito et al. 2004, Miyakawa & Yamaguchi 1981). These viruses have properties in common with SDV (Tanaka & Yamada 1972, Iwanami 2010, Hyun et al. 2020, Usugi et al. 1986) and they have been classified as SDV strains; however, NIMV and HV are apparently distinct from SDV (Iwanami 2010, Iwanami et al. 2001, Ito et al. 2004, Yan et al. 2020).

i) Properties of CiMV

CiMV typically induces the development of green blotches and ring-shaped spots on the fruit rind during coloration as well as delayed coloring of the affected areas (Miyakawa & Yamaguchi 1981, Yamada & Tanaka 1968). The disease caused by CiMV was considered to be an unknown fruit disorder until the late 1950s, when it was first described as a viral disease (Yamamoto & Yamaguchi 1980). Disease symptoms have been detected on the fruit of several species, including satsuma mandarin, lemon [*Citrus limon* (L.) Osbeck], and kinkoji (*Citrus obovoidea* hort. ex Tanaka) (Miyakawa & Yamaguchi 1981). Foliar symptoms induced by CiMV are similar to those caused by SDV (Miyakawa & Yamaguchi 1981). A recent study on Jeju Island confirmed that fruit on CiMV-infected trees, while having dark blue speckles or ringspots on the rinds as well as brown oil glands in the infected spots during the rind-coloring period of satsuma mandarin and hybrid cultivars (e.g., "Setoka" and "Kiyomi") (Hyun et al. 2020), have relatively thick, hard, and swollen rinds and low sugar concentrations. This previous study indicated CiMV induces similar symptoms in Korea and Japan. Similar to SDV, CiMV is easily transmitted to many herbaceous plants, including sesame and *P. floridana*, via mechanical inoculation (Usugi et al. 1986).

CiMV is widely distributed in Wakayama Prefecture in Japan (Inuma & Tomimura 2016). The type isolate Ci-968 (Iwanami et al. 1993a) was collected from a satsuma mandarin tree in Wakayama Prefecture. CiMV is often found in other parts of Japan (Iwanami et al. 1991a, 1993a; Miyoshi S. et al. 2020) as well as in Korea and China (Hyun et al. 2017, Yan et al. 2020). To date, CiMV has not been detected in Turkey. A comparison of a partial RNA2 nucleotide sequence revealed a 95.5%-96.2% sequence identity between Korean CiMV isolates and the Japanese isolate Ci-968 (Hyun et al. 2017). In Japan, some novel CiMV isolates that are serologically distinct from Ci-968 have been reported (Iwanami et al. 1993a, Miyoshi et al. 2020). For example, Az-1 is serologically distinct from Ci-968, but its nucleotide sequence is highly homologous to that of Ci-968 (Iwanami et al. 1993a, Iwanami et al. 2001). Isolates (e.g., B291) that are serologically similar to Az-1 are widespread and seriously affect satsuma mandarin cultivation in Ehime Prefecture, resulting in considerable economic losses (Miyoshi et al. 2020). At the amino acid level, a Chinese isolate (CiMV-SE1) was revealed to be the most similar to Az-1 (Yan et al. 2020). These findings indicate that Az-1 is not an orphan isolate, but it represents a serologically distinct CiMV strain (or more precisely, it should be referred to as a sub-strain of the CiMV strain of SDV). An infection of sweet orange by another isolate (LB-1)

Table 1. Strains, sub-strains, and isolates of satsuma dwarf virus, apple stem grooving virus (citrus tatter leaf virus), and citrus tristeza virus referred in this paper[a]

Order	Family	Sub-family	Genus	Subgenus	Species	Strain	Sub-strain	Isolate	References
Picornavirales	*Secoviridae*	N.A.[b]	*Sadwavirus*	*Satsumavirus*	*Satsuma dwarf virus*	SDV strain	N.A.	S-58	Iwanami (2010)
						CiMV strain	CiMV sub-strain	Ci-968	Iwanami (2010)
								GTC1	Yan et al. (2020)
								LB-1	Iwanami (2010)
							Az-1 sub-strain	Az-1	Iwanami (2010)
								B291	Miyoshi et al. (2020)
								SE1	Yan et al. (2020)
							NDV sub-strain	ND-1	Iwanami (2010)
						NIMV strain	N.A.	NI-1	Iwanami (2010)
								EH1	Yan et al. (2020)
						HV strain	N.A.	KNO2	Ito et al. (2004)
Tymovirales	*Betaflexiviridae*	*Trivirinae*	*Capillovirus*		*Apple stem grooving virus*	CTLV strain	N.A	N297	Iwanami et al. (1991b)
								MTH	Song et al. (2015)
Martellivirales	*Closteroviridae*	N.A.	*Closterovirus*		*Citrus tristeza virus*		N.A.	T36	Fuchs et al. (2020)
								M-15A	Ieki et al. (1997)
								M-16A	Ieki et al. (1997)

[a] Only isolates referred in this paper are listed. Some strains (CiMV, NIMV, NDV) and all sub-strains shown here are tentative, and more studies and discussions are needed before establishment of these lower taxa.

[b] Not available: such taxa are not established.

results in unique blotchy leaves (Iwanami et al. 1991a). Typical mosaic symptoms have never been observed on LB-1-infected sweet orange fruit in fields. LB-1 is also serologically distinct from SDV-S-58 and CiMV-Ci968 (Iwanami et al. 1991a). In terms of the amino acid sequence, CiMV-GTC1, which is a Chinese isolate, is the most similar to LB-1 (Yan et al. 2020). These results suggest that LB-1 is also another sub-strain of the CiMV strain of SDV. A third sub-strain (NDV) is described in more detail in the NDV subsection. A previous study revealed the genetic diversity of a heterogeneous CiMV population within a single citrus tree (Ito et al. 2007). Thus, CiMV is widely distributed in Japan, Korea, and China, with diverse variant isolates. Care should be taken when reviewing old literature regarding CiMV because some Indian researchers referred to another bacilliform virus as CiMV until the 1990s. The Indian virus was correctly designated as citrus yellow mosaic virus in the 2000s (Baranwal et al. 2005).

ii) Properties of NIMV

NIMV infections of navel orange typically lead to mottling and the development of large and diffuse chlorotic blotches on leaves (Miyakawa & Yamaguchi 1981, Yamada & Tanaka 1968). The foliar symptoms characteristically develop and persist on all flushes, whereas the foliar symptoms caused by SDV are restricted to spring flushes (Miyakawa & Yamaguchi 1981). Compared with normal trees, severely infected trees yield fewer and smaller fruits (Miyakawa & Yamaguchi 1981, Yamada & Tanaka 1968). Similar to SDV, NIMV is easily transmitted to many herbaceous plants, including sesame and *P. floridana*, through mechanical inoculation (Tanaka & Imada 1976).

Navel orange infectious mottling was a disease affecting navel orange grown in a limited area of Katsuragi, Wakayama Prefecture as early as 1930. It was initially believed to be caused by a virus related to citrus variegation virus (CVV) (Yamada & Tanaka 1968). A subsequent electron microscopy analysis of virions indicated that NIMV has polyhedral particles that differ morphologically from those of CVV (Tanaka & Imada 1976). NIMV was long considered to be endemic in a few trees in Katsuragi (Miyakawa & Yamaguchi 1981). The type isolate NI-1 was collected from one of these trees. However, a recent study detected another NIMV isolate (NIMV-EH1) in China (Yan et al. 2020). Furthermore, a phylogenetic analysis revealed the genetic diversity among NIMV isolates (Yan et al. 2020). The nucleotide sequences of NIMV isolates are distinct from those of SDV strains; NIMV represents a distinct strain of SDV (Iwanami 2010). There are currently no reports of NIMV in Korea or Turkey.

iii) Properties of NDV

Natsudaidai is a late-maturing species of citrus that produces large fruit with a pleasant sour taste. A virus-like disease (natsudaidai dwarf) was first observed on natsudaidai plants grown in Hagi, Yamaguchi Prefecture in the 1950s. An examination of purified virions and the results of agar gel diffusion tests using SDV and CiMV antisera revealed relationships among SDV, CiMV, and NDV (Imada & Narisawa 1979). To date, NDV has been detected only in Hagi, Yamaguchi Prefecture, Japan. There has been no reported NDV outbreaks elsewhere in Japan or in other countries. The type isolate ND-1 was collected from infected plant material in Hagi. An earlier comparison of viral genome sequences indicated that NDV is a sub-strain of the CiMV strain of SDV (Iwanami et al. 2001). The diversity among NDV isolates has yet to be explored.

The initial symptoms of an NDV infection of *C. natsudaidai* typically include the vein-clearing of young expanding leaves, whereas the later symptoms include the mottling, curling, and crinkling of newly expanded leaves in the spring (Miyakawa & Yamaguchi 1981, Yamada & Tanaka 1968). Foliar symptoms vary annually and may appear on summer and autumn flushes (Miyakawa & Yamaguchi 1981). In graft-transmission tests, NDV induces specific foliar symptoms on rough lemon (*Citrus jambhiri* Lush.) (Miyakawa & Yamaguchi 1981). Similar to SDV, NDV is easily transmitted to many herbaceous plants, including sesame and *P. floridana*, via mechanical inoculation (Tanaka & Imada 1974).

iv) Properties of HV

Hyuganatsu, a chance seedling found in Miyazaki Prefecture more than 150 years ago, is a late-season citrus species. Hyuganatsu is now produced as a special product in specific regions, including Miyazaki, Kochi, and Shizuoka Prefectures. A viral isolate (KNO2) that was serologically related to SDV was sampled from hyuganatsu and then tentatively designated as HV (Ito et al. 2004). Whether HV induces visible symptoms on hyuganatsu remains to be determined (Ito et al. 2004). HV, where the diversity among HV isolates has not been explored, and because of the more than 75% homology of the amino acid sequences being encoded by the coat protein genes and RNA-dependent RNA polymerase gene, has been classified as a distinct SDV strain (i.e., in addition to CiMV and NIMV) (Iwanami 2010).

4. Diagnostics

(1) ELISA and immunochromatography test

Because SDV, CiMV, NDV, and HV are serologically related, they can be detected on citrus plants using antiserum raised against one of these viruses (e.g.,

SDV) (Ito et al. 2004, Iwanami et al. 1993a, Iwanami 2010). However, a specific antiserum is needed to detect NIMV, which is serologically distinct from these viruses (Iwanami 2010). Many lines of polyclonal and monoclonal antibodies targeting SDV and CiMV have been raised in Japan and elsewhere; they have been used for serological diagnoses, typically in DAS-ELISA platforms (Hirashima et al. 1990, 1991, 1994; Nozu et al. 1986; Usugi & Tsuchizaki 1982; Wu et al. 2012; Zhou et al. 1996). The anti-CiMV rabbit monoclonal antibodies developed recently may be very useful for detecting CiMV in infected plant material (Miyoshi et al. 2020).

One drawback of ELISA is the need for expensive equipment (e.g., microplate reader), many reagents, time, and skilled technicians, where, in contrast, an immunochromatography test is very simple and can be performed by anyone, including nursery workers and farmers. An immunochromatography test kit for SDV, remarkably enabling the all-season diagnosis of SDV using young shoots and flower buds in the spring, fruit rinds in the summer and autumn, and the rinds of stored fruit and sprouts from scions in the winter (Kato et al. 2014), was developed (Kusano et al. 2007, Iwanami 2010) and used for detecting the virus under field conditions (Kato et al. 2014). Furthermore, a recent report suggested that the immunochromatography test kit for SDV is more suitable for large-scale diagnoses than a DAS-ELISA (Kato et al. 2020). An immunochromatography test kit using monoclonal antibodies specific for NIMV was also developed (Kusano & Asakuma 2009).

(2) PCR-based detection

In principle, reverse transcription PCR (RT-PCR) is more sensitive than serological methods for detecting viruses, making it indispensable for testing important materials (e.g., mother trees). A primer set (FW146/RV488) was designed according to conserved sequences among SDV, CiMV, NDV, and NIMV isolates. An RT-PCR using this primer set apparently detected most, if not all, SDV, CiMV, NDV, and NIMV isolates as well as HV (Iwanami 2010). In Korea, a multiplex RT-PCR assay using these primer sets was developed to detect SDV, CiMV, CTLV, and CTV (Hyun et al. 2017). A universal primer set (uSDVup/uSDV) was also developed and used to clarify the genetic diversity among these SDV variants (Shimizu et al. 2011). A field survey involving the multiplex RT-PCR assay revealed that 35.2% of 775 trees in 155 orchards on Jeju Island were infected with SDV or CiMV (Hyun et al. 2017).

5. Control

(1) Production of virus-free mother trees

The primary source of SDV is contaminated budwoods and nursery trees. Accordingly, the production and maintenance of virus-free mother trees are critical for controlling SDV infections. For most citrus cultivars, natural SDV-free mother trees are available in Japan, with many of them being used for vegetative propagation. If all available mother trees are infected, SDV must be eliminated. For example, SDV is readily eliminated by grafting seedlings of trifoliate orange [*Poncirus trifoliata* (L.) Raf.] with a shoot-tip that is less than 0.3 mm long after heat treatment at 36°C / 26°C day/night for about 30 days (Hirashima & Noguchi 1991). The success rate of shoot-tip grafting was increased by using Troyer citrange as a rootstock (Morita & Koizumi 1995). The elimination of SDV by combining semi-micrografting and heat treatment was also reported (Ohta et al. 2016). Compared with shoot-tip grafting, semi-micrografting is easier, but it eliminates SDV less efficiently (Ohta et al. 2011). This drawback may be alleviated by applying an antiviral agent (e.g., foscarnet) (Ohta et al. 2011). In China, it is reportedly difficult to remove SDV from infected material by shoot-tip grafting or by a heat treatment alone, but the virus can be completely eliminated by combining a heat treatment at 40°C (day) / 30°C (night) for 7-43 days with shoot-tip grafting (Zhou et al. 1994). Similar results were also obtained for SDV in Korea (Kim et al. 2005). Shoot-tip grafting was also applied to eliminate SDV from citrus trees in Turkey (Göral et al. 1993).

CiMV can be efficiently eliminated from citrus plants, where there are no reports describing the elimination of NIMV, NDV, and HV, likely because mother trees free of these viruses have always been available, by this shoot-tip grafting combined with heat treatment under the same conditions as those used for removing SDV (Hirashima & Noguchi 1991).

(2) Prevention of soil transmission

Soil fumigation by methyl bromide, ammonium N-methyl-dithiocarbamate, 1,3-dichloropropene, and trichloronitromethane (chloropicrin), which in tablet form has been registered in Japan for controlling SDV infections (Green Japan 2021), and the efficacy of which has been confirmed by another field trial (Kageyama et al. 2011), can prevent the infection of replanted citrus trees in a commercial field (Katagi & Ushiyama 1990). In Japan, citrus trees are typically grown in narrow and irregularly shaped terraces on steep slopes. Thus, the complete sealing of the soil surface, which is crucial for an effective fumigation, might be difficult in these fields. The re-occurrence of an SDV infection was observed in about 20% of the replanted satsuma mandarin trees 8 years after infected trees were removed and the field was fumigated with chloropicrin (Yamaguchi et al. 1981). Interestingly, the infection rate was much lower

(less than 4%) in a field where a bulldozer was used for replanting than in a field where only hand-tools were used (Yamaguchi et al. 1981). The efficacy of a bulldozer-based replanting method and the underlying reasons for the decreased infection rate (e.g., bulldozer effects on the soil) should be investigated in future field trials.

SDV infects a wide range of citrus and citrus-related species (Miyakawa 1969). Resistant citrus cultivars are rare, with only natsudaidai reported to be resistant to SDV (Yamaguchi 1984). More specifically, natsudaidai seedlings tested negative for SDV in a DAS-ELISA performed 10 months after a graft-inoculation. However, these results have not been reproduced by other researchers and the reported resistance of natsudaidai will need to be verified. Nevertheless, the resistance to SDV is strain-specific at best, considering that NDV, which is an SDV strain, readily infects natsudaidai. An earlier study demonstrated that five natsudaidai nucellar seedlings remained SDV-free even 11 years after they were transplanted in an SDV-infested field, whereas five trifoliate orange nucellar seedlings became infected within 9 years (Kageyama et al. 2011). Natsudaidai is too vigorous to be used as a citrus rootstock in most cases. To overcome this problem, a series of hybrids derived from a cross between natsudaidai and trifoliate orange was produced by the Okitsu Branch, Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries, Japan (currently the institute is a subsidiary of the National Agriculture and Food Research Organization, Japan). Some of these hybrids with a moderate tree vigor were SDV-free 11 years after they were transplanted in an SDV-infested field (Kageyama et al. 2011). Although promising, these results were obtained from hybrids that were tested without replicates. Therefore, additional field tests will need to be conducted to evaluate the utility of these hybrids as SDV-resistant rootstocks.

Trifoliate orange plants were transformed with a binary vector containing the capsid polyprotein (pCP) gene of CiMV via *Agrobacterium tumefaciens*. Some of the generated transgenic lines were moderately to highly resistant to CiMV infections (Iwanami et al. 2004). Their tolerance under natural infection conditions should be confirmed in future studies.

CTLV

1. Occurrence

(1) Occurrence in Japan

CTLV is sometimes found in satsuma mandarin, ponkan mandarin (*Citrus reticulata* Blanco), and tankan tangor (*Citrus tankan* Hayata) lines as well as in other citrus cultivars. A high percentage of ponkan mandarin trees, particularly “Kosho-type” lines, and most tankan tangor lines introduced from colonial Taiwan were infected with CTLV (Miyakawa & Yamaguchi 1981). CTLV induces a bud-union disorder in citrus cultivars grafted on trifoliate orange rootstock, making it one of the most serious viruses affecting citrus production in Japan, where trifoliate orange is widely used as a rootstock because of its cold hardiness and CTV tolerance.

(2) Occurrence around Japan

CTLV is a serious threat anywhere trifoliate orange and its hybrids, such as Rusk citrange [*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.] are used as a rootstock (Fig. 1B). CTLV latently infects citrus on other rootstocks, including sunki (*Citrus sunki* Hort. ex Tanaka) and yuzu (*Citrus junos* Siebold ex Tanaka). CTLV was found to be widespread in Taiwan and mainland China, and it has also been detected on Jeju Island, Korea (Hyun et al. 2017, Su & Cheon 1984, Zhang et al. 1988). Notably, although ponkan and tankan trees with sunki rootstocks in orchards appeared to be healthy, they were commonly infected with CTLV (Su & Cheon 1984). This may help to explain why contaminated ponkan and tankan trees were exported from Taiwan in the past.

2. Biological and molecular biological properties

ASGV infects a wide range of horticultural crops, including most citrus cultivars as well as pome fruit, kiwifruit, bamboo, and lily (Massart et al. 2011, Wang et al. 2018, Bhardwaj et al. 2017, Inouye et al. 1979). CTLV, which is an ASGV strain, was first detected following the grafting of *Citrus excelsa* Wester to symptomless Meyer lemon trees in California, USA (Wallace & Drake 1962). The infected *C. excelsa* leaves were deformed and had ragged margins, from which the tentative virus name (i.e., tatter leaf virus) was derived. The virus was subsequently renamed as citrange stunt virus (CSV) (Wallace & Drake 1968). Since then, both CTLV and CSV have been used in published articles on citrus diseases. In 1993, the striking similarities in the nucleotide sequence and genome organization between CTLV and ASGV were reported (Yoshikawa et al. 1993), after which CTLV was considered to be an ASGV strain (Magome et al. 1997). In hindsight, ASGV maybe should have been designated as a CTLV strain, but this possibility was not considered. Notably, CTLV, which may be transmitted to some herbaceous plants, including cowpea, kidney bean, and *Chenopodium quinoa*, and then back to citrus plants via mechanical inoculation (Miyakawa & Yamaguchi 1981), and ASGV have been used interchangeably in many published articles (Tan et al. 2019, Cowell et al. 2018). The propagation of contaminated scions is apparently the major cause of CTLV spread in fields, with no known vector under

natural conditions (Miyakawa & Yamaguchi 1981).

CTLV has flexuous elongated thread-like helical virions that are approximately 600-700 nm long and 12 nm in diameter (Fig. 1E). Additionally, it has a monopartite genome comprising a 6.5-kb single-stranded (+)-sense RNA sequence with a 5′ cap and a polyadenylated 3′ terminal. The genomic RNA contains two overlapping open reading frames, namely ORF1 (6.3 kb) and ORF2 (1.0 kb), which encode proteins with a molecular mass of 241 and 36 kDa, respectively (Yoshikawa & Takahashi 1992).

3. Taxonomy and strains

(1) Taxonomy

ASGV belongs to the genus *Capillovirus* of the subfamily *Trivirinae* of the family *Betaflexiviridae* of the order *Tymovirales*. As discussed in the previous section, CTLV is considered to be an ASGV strain because of the similarities in the genomic RNA sequences (Table 1).

(2) Strains

There is relatively little diversity in the pathogenicity of CTLV among citrus species, with most isolates similarly inducing typical bud-union disorders in citrus cultivars grafted on trifoliate orange rootstock. In a previous study, an ASGV originally isolated from apple induced a bud-union disorder in a citrus cultivar grafted on trifoliate orange rootstock (Iwanami et al. 1991b). In the same study, a host-range test involving herbaceous plants indicated that the CTLV type isolate in Japan (N297) can induce systemic infections of kidney bean (*Phaseolus vulgaris* L.), whereas four other isolates can only induce local lesions on the inoculated leaves (Iwanami et al. 1991b). This finding suggests there may be some CTLV biological strains. A recent analysis of the 28 available full genome sequences of CTLV and ASGV citrus isolates revealed the remarkable diversity in the genomic RNA sequences, which separated the isolates in four clusters in a phylogenetic tree (Tan et al. 2019). Another study in China, indicating that some ASGV isolates and CTLV isolates form a genetically related group irrelevant to their host plants (Song et al. 2015), determined that CTLV-MTH isolated from citrus plants is more closely related to ASGV isolates than to CTLV isolates. A recent study on an ASGV isolate from kiwifruit determined that it varies substantially from other ASGV isolates from kiwifruit (i.e., only 79.5%-82.4% genome sequence identity), implying it is likely a novel variant (Wang et al. 2018). The infectivity of this variant among citrus species remains to be explored.

4. Diagnostics

(1) ELISA and immunochromatography test

The CTLV K-1 isolate in infected citrus leaves can be detected by an ELISA using horseradish peroxidase-conjugated IgG (HRP-ELISA); this technique is widely applicable for detecting CTLV in citrus plants because CTLV isolates are apparently serologically homologous (Kawai & Nishio 1990). To increase the utility of this assay, the sample preparation procedure was modified to decrease costs and the time required to perform the assay (Kusano et al. 2009). More recently, a simple and rapid immunochromatographic assay for detecting CTLV in citrus plants was developed following the production of several lines of specific monoclonal antibodies (Kusano et al. 2014). On the basis of this assay, a commercially available diagnostic kit, SDV/ASGV Chromato (immunochromatography) that simultaneously detects both SDV and CTLV (ASGV) were produced (Mizuho Meddy 2021).

(2) PCR-based detection

A multiplex RT-PCR assay for the simultaneous detection of six citrus viroids and CTLV in citrus plants was developed and used to investigate the distribution of these pathogens in 217 citrus trees grown in Japan (Ito et al. 2002a, Ito et al. 2003). The results indicated that CTLV occurs only occasionally in Japan (Ito et al. 2003). Another RT-PCR assay, which was developed in Australia, revealed the nucleotide sequences of Australian and Japanese isolates are highly homologous (Hailstones et al. 2000). Additionally, RT-PCR primer sets PP2-1, CTLV-2013, and CTV-po were designed for the simultaneous detection of SDV/CiMV, CTLV, and CTV, respectively, on Jeju Island, Korea. A field survey using this detection system did not detect CTLV in any of 775 examined trees in 155 orchards on Jeju Island (Hyun et al. 2017). To improve sensitivity, a one-step immunocapture RT-PCR (One-Step IC-RT-PCR) assay was developed. This assay requires only one PCR tube, and in contrast to ELISA can be used to detect CTLV in mature leaves (Kusano & Ibi 2003), where it can decrease the required labor and minimize the risk of cross contamination. An immunocapture RT-PCR method was also used by American researchers to analyze CTLV in the original Meyer lemon host (Hilf 2008).

5. Control

(1) Production of virus-free mother trees

After CTLV-free mother trees are established, CTLV infections can be avoided as long as scions are obtained from these mother trees, as CTLV is transmitted mostly through vegetative propagation, and no other means of transmission has been known. CTLV may be eliminated from infected citrus plants by a heat treatment at 40°C (day) / 30°C (night) for more than 60 days (Calavan et al. 1972). Unfortunately, most satsuma mandarin

cultivars cannot survive such a long heat treatment period. To address this problem, a method involving shoot-tip grafting following a 30-day heat treatment was established (Koizumi 1984). Using this method, CTLV can be efficiently eliminated from most citrus cultivars. One drawback of this method is that it requires a skilled technician to perform the shoot-tip grafting step. Because of limited funding, it is becoming increasingly difficult to employ a suitably skilled technician full-time. This has necessitated the development of simpler methods for eliminating CTLV. For example, antiviral agents have been used to produce CTLV-free buds from infected trees (Iwanami & Ieki 1994). More specifically, in a previous study, the growing shoots of several citrus cultivars infected with various sources of CTLV were sprayed with a ribavirin aqueous solution (500 ppm) at 1-week intervals for several weeks, where CTLV resistant to the ribavirin treatment was eliminated by combining the ribavirin treatment with a heat treatment (Iwanami & Ieki 1994). This aforementioned treatment efficiently eliminated all but one source of CTLV from the buds. A shoot-tip culture using ribavirin can also efficiently eliminate CTLV from infected citrus cultivars (Iwanami et al. 1993c).

CTV

1. Occurrence

(1) Occurrence in Japan

The wide distribution of CTV in Japan was first detected in the 1950s. Later, stem-pitting diseases associated with CTV were reported for hassaku (*Citrus hassaku* hort. ex Yu. Tanaka), natsudaidai, yuzu, and iyo (*Citrus iyo* hort. ex Tanaka) (Miyakawa & Yamaguchi 1981, Fig. 1C). In one field survey, most of the examined satsuma mandarin trees were infected with CTV, but lacked clear symptoms (Yamada et al. 1979). In Japan, the Rikuzen-Takata/Ofunato areas in Iwate Prefecture, which are located at the northern limit of the Japanese citrus cultivation region, are the only known citrus-producing areas in which many old plants that derived from seeds are free of CTV (Nagaoka-Nakazono et al. 2020).

(2) Occurrence around Japan

CTV is widely distributed in Asia, Oceania, Africa, Europe, North America, and South America (European Food Safety Authority 2019). CTV is commonly found throughout China (Zhou 2018). Researchers have speculated that CTV was present in ancient China and Japan (Bar-Joseph et al. 1989). Through the movement of infected plants and vectors, CTV in China was likely sequentially introduced to Japan, the Philippines, India, Australia, and South Africa (Roistacher et al. 2010). In Taiwan, the diversity among cultivars and long-term CTV infections have resulted in a CTV infection rate exceeding 90% in citrus fields (Lin et al. 2018). In Korea, CTV is widely distributed among satsuma mandarin and yuzu fields (Kim et al. 2000, Kim et al. 2006). One survey on Jeju Island revealed that, whereas Dangyooja, a mandarin accession native to Jeju Island, was free of CTV (Oh et al. 1999), many yuzu, "Koyomi" and "Shiranui" mandarin field-grown trees that were apparently introduced from Japan were infected with CTV.

2. Biological and molecular biological properties

CTV infections lead to the following three major symptoms: stem-pitting (SP), which occurs in grapefruit, lime, pummelo, and sweet orange; seedling yellows (SY), which occurs in sour orange and lemon; and decline, which results in the death of sweet orange, mandarin, and grapefruit trees with a sour orange rootstock (Roistacher et al. 2010, Dawson et al. 2013). Growing satsuma mandarin on trifoliate orange rootstocks is a major cultivation practice in Japan. CTV does not induce severe SP in satsuma mandarin. Additionally, CTV does not cause SY in trifoliate orange seedlings or decline in satsuma mandarin on trifoliate orange rootstocks. Thus, there have been no SP, SY, and decline epidemics in the satsuma mandarin-growing regions in Japan. However, some early-maturing satsuma mandarin cultivars are apparently more susceptible to CTV than common satsuma mandarin (Koizumi et al. 1989).

The effect of SP on Japanese citrus species was observed using hassaku, yuzu, natsudaidai, and sanbokan (*Citrus sulcata* Hort. Ex Tanaka) plants in the 1960s and 1970s (Miyakawa & Yamaguchi 1981). Navel orange and iyo were also severely affected (Miyakawa & Yamaguchi 1981). A 1978 survey in Hiroshima Prefecture indicated that 38% of hassaku plants were affected by SP (Miyakawa & Yamaguchi 1981).

CTV is naturally transmitted in a semi-persistent manner primarily by the brown citrus aphid [*Toxoptera citricidus* (Kirkaldy)], which is widely distributed in Japan, including in the northernmost citrus-producing area of Iwate Prefecture (Nagaoka-Nakazono et al. 2020). CTV infects only citrus and citrus relatives under natural conditions (Dawson et al. 2013), although some *Passiflora gracilis* (one of the passion flowers) plants have been infected under experimental conditions (Müller et al. 1974).

CTV has long flexuous thread-like virions that primarily infect phloem-associated cells (Folimonova 2020, Fig. 1F). The viral genome consists of a 19.3-kb single-stranded (+)-sense RNA sequence (Dawson

et al. 2013), which includes 12 open reading frames that potentially encode at least 19 proteins (Dawson et al. 2013).

3. Taxonomy and strains

(1) Taxonomy

CTV belongs to the genus *Closterovirus* in the family *Closteroviridae* in the order *Martellivirales*. Its exemplar isolate is T36, which was isolated in Florida, USA (Fuchs et al. 2020, Table 1).

(2) Strains

Miyakawa (1987) reported that both SP and SY are common in field-grown citrus trees infected with CTV in Japan. In addition to biological variations, there are published articles describing the serological and genetic diversity of CTV isolates in Japan (Kano et al. 1991, Kano et al. 1998, Suastica et al. 2001), with some CTV sources reportedly not inducing the three main disease symptoms (i.e., SP, SY, and decline), which implies they comprise only mild CTV strains. Many mild strains have been found in Japan after Sasaki (1967) first detected a mild CTV strain in Hassaku trees, some of which have been successfully applied to provide cross protection in fields (Ieki et al. 1997). Although the genotyping of the Japanese strains according to this standardized method has not been completed, the standardization of the classification of CTV genotypes has been proposed (Harper 2013).

4. Diagnostics

(1) ELISA and immunochromatography test

A DAS-ELISA for CTV using polyclonal antibodies was developed in Japan in the early 1980s and it has been extensively used since then (Koizumi & Kuhara 1984). A commercially available immunochromatography test kit for CTV has been developed. Additionally, the results of a DAS-ELISA confirmed that all tested Japanese CTV isolates can be detected using polyclonal antisera from Florida, USA and Japan (Kano et al. 1991). In contrast, the serological diversity among Japanese CTV isolates was revealed on the basis of the reactions to the monoclonal antibodies MCA13 and 3DF1 (Kano et al. 1991). This diversity should be considered when using detection kits involving monoclonal antibodies.

(2) PCR-based detection

There has been relatively little research regarding the application of PCR-based techniques for detecting CTV in fields in Japan, presumably because easier and more cost-effective serology-based detection kits are widely available. A conventional RT-PCR assay was recently used to detect CTV in trees grown in the northernmost citrus-producing areas in Japan (Nagaoka-Nakazono et al. 2020), with selecting appropriate primer sets being critical for detecting the target CTV strains, because of the substantial variability in the genome sequences among Japanese CTV isolates (Kano et al. 1998).

5. Control

(1) Establishment of disease-free mother trees and application of mild strains

Because CTV and its vector are widely distributed in Japan, the basic approach to controlling CTV infections is the establishment of disease-free mother trees and the application of mild strains to prevent infections by severe strains. Hassaku growers were encouraged to take scions from trees with healthy buds that apparently carried mild CTV strains. This practice was used extensively from the 1960s to the 1980s (Miyakawa & Yamaguchi 1981). CTV was readily eliminated from infected trees by semi-micrografting, which is a simplified form of shoot-tip grafting (Takahara et al. 1986). Field-grown navel orange trees that were pre-inoculated with the mild strains M-16A and M-15A after eliminating the indigenous wild CTV by a heat treatment increased yields by about 1.5-times 7-9 years after the challenge inoculation (Ieki et al. 1997). Furthermore, CTV-free yuzu lines were selected and planted in a field without a pre-inoculation with mild strains in the northernmost citrus-producing areas in Iwate Prefecture. The yuzu trees were still free of CTV several years after they were transplanted (Nagaoka-Nakazono 2020). Brown citrus aphids were only sporadically detected in the examined areas, but they were undetectable during the field trial (Nagaoka-Nakazono 2020). A similar control strategy might be applicable in cool regions (e.g., northern China and Korea) as well as at high altitudes in Taiwan, the Philippines, and other tropical areas, where the vector insect is present only occasionally and its population is relatively low.

Conclusion

SDV and some isolates of CTV were apparently indigenous to Japan, other CTV isolates were brought from ancient China and CTLV was brought from colonial Taiwan. SDV was obviously exported from Japan to other areas. These viruses have been characterized and the countermeasures have been developed, where, as globalization proceeds, there will be increased threat of dissemination of these viruses. To deal with this problem, further research should be promoted by international collaboration, and the insight mentioned should be shared by all related countries.

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