Plant Viroid Diseases Occurring in Japan

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Viroids are the small pathogenic RNA molecules known to be the causative agents of several plant diseases[1]. The viroid diseases are of worldwide occurrence and have caused serious economic losses in the temperate and subtropical climates[35]. Five viroids attacking several crops have been identified in Japan so far. This paper attempts to summarize their natural occurrence, biological properties, symptoms, mode of transmission, indexings and control measures.

Occurrence of viroid diseases

1) Citrus exocortis disease

Exocortis was first recognized in 1963 in the field at Okitsu Branch of Fruit Tree Research Station in Shizuoka Prefecture[45]. The affected trees were severely stunted and exhibited characteristic bark shelling on the trifoliate orange rootstocks. Since then systematic surveys were made by the Ethrog citron test or diagnosis of the symptoms developed on the trifoliate orange rootstocks. The indexings revealed that exocortis was seen on citrus plantings in various prefectural experiment stations as well as some commercial plantings in several prefectures[43,44,48,49,53,54]. Recently, six groups of the variants were isolated according to their biological properties differentiated on the indicators, such as Ethrog citron and *Gynura aurantiaca*[5].

2) Hop stunt disease

Commercial hop cultivation in Japan is mostly distributed in the northern part of the mainland of Japan such as Tohoku District and in Hokkaido. As early as in 1952 hop plants showing stunt syndrome were observed in several hop gardens in Fukushima Prefecture, and in 1959 occurrence of the similar syndrome was also noticed in Nagano Prefecture. Hop stunt disease was described in Japan for the first time by Yamamoto et al.[57]. It was presented that the disease was assumed to be of viral etiology, however, no causal viral particles have been detected in the diseased plants, Sasaki and Shikata[30,31] in 1977 have established the detecting procedure using cucumber as an indicator plant and provided evidence that hop stunt was a viroid-incited disease. In their investigations, viroid nature of the disease agent was suggested by the observations including low sedimentation rate, sensitivity to treatment with ribonuclease and apparent absence of virus particles in infected tissues. Takahashi[34] confirmed these results and offered the proof that characteristic stunt syndrome of hop plants was caused by a low molecular weight RNA. In hop stunt viroid (HSV)-infected hop cones, the lupulin glands are distributed most abundantly on the bracteoles and the perianths, and their numbers are reduced by at least 60% of that in HSV-free control[15]. The content of α-acid was half to one third of that of HSV-free hop cones[15,57]. At present, the area of infested hop gardens has gradually decreased as a result of removal of infected root systems and of replanting with certified, HSV-free hop plants.

3) Chrysanthemum stunt disease

Occurrence of chrysanthemum stunt was first recognized in Shizuoka Prefecture in 1977[20]. The disease is known to be widely

distributed in chrysanthemum-growing regions. The disease agent was usually detected by polyacrylamide gel electrophoresis (PAGE) analysis and by bioassay using chrysanthemum cv. Mistletoe[2,3].

4) Apple scar skin disease

The disease was first described by Ohtsuka[21] in 1935 and named "Manchurian sabika-byo" from the results of the transmission experiments by grafting[22]. In Japan, occurrence of the scar skin or dapple apple was also reported[32,47,56]. The foliage and trunk of the diseased trees appear normal, but symptoms being found only on the fruits. From the graft transmission experiments, Yamaguchi and Yanase[55] suggested evidence that scar skin and dapple apple diseases were caused by the same agent. It was elucidated by Koganezawa et al.[9] in 1982 that low molecular weight RNA (ASSARNA 1) isolated from the diseased fruits has the properties similar to the known viroids. Later, it was confirmed that ASSARNA 1 was transmitted to apple seedlings[7,8] and developed characteristic symptoms on the fruit of back-inoculated tree. However, mechanical transmission to herbaceous hosts of the causal viroid has not yet been reported so far.

5) Plum dapple disease

A malady showing "dapple" syndrome in plum fruit was noted in Yamanashi Prefecture as early as in 1968. The affected trees produce dapple fruits with faint reddish and chlorotic blotches, but no visible symptom appears on any leaves or stems. Terai[46] in 1985 proposed to be named plum dapple disease because of the graft-transmissibility. The causal agent of the disease was low molecular weight RNA (ca. 300 nucleotides) and hybridized weakly with complementary DNA (cDNA) of HSV-cucumber isolate[27].

Biological properties of viroids

The molecular weight of the known viroids is about 80,000 to 125,000 daltons and their nucleotide chain is only 246–371 nucleotides long[35]. Among several viroids isolated in Japan, HSV-cDNA was first cloned and the complete sequence consisting of 297 nucleotides was determined[18]. Since then some HSV isolates belonging to the HSV group have been described (Table 1)[22a,24,26,27,29]. Electron microscopy indicates native forms of HSV as short rod-like structures and denatured forms as covalently closed circular molecules[17,23]. The mechanisms of viroid replication were not fully elucidated, but it is certain that viroids depend upon the nuclear enzymes already present in the host plants for their replication. In fact, infectious HSV is present in isolated nuclei from the infected leaves[11]. From the tests on inhibitor effects, it was evident that α-amanitin specifically inhibits replication of HSV-RNA, thus suggesting a role of nuclear DNA-directed RNA polymerase II[59]. Double stranded DNA fragments containing multimeric HSV-cDNA and their *in vitro* transcripts were infectious[11,19]. Two and four unit tandemly repeated plus strand RNAs were indeed infectious, but one

Table 1. Molecular size of viroids found in Japan

Viroid	Nucleotide chain length
PSTV group	
CEV	[371**]
CSV	[354**] [356***]
HSV group	
HSV	297[18]
HSV-cucumber isolate*	303[24]
HSV-grapevine isolate	297[26]
HSV-citrus isolate	302[22a,29]
PDV	ca. 300[27]
Unknown group	
ASSV	330[3a]

* Cucumber pale fruit viroid found in the Netherlands.

** From: Gross, H. J. et al.: *Eur. J. Biochem.*, **121**, 249–257 (1982).

*** From: Haseloff, J. & Symons, R. H.: *Nucleic Acids Res.*, **9**, 2741–2752 (1981).

PSTV: potato spindle tuber viroid, CEV: citrus exocortis viroid, CSV: chrysanthemum stunt viroid, HSV: hop stunt viroid, PDV: plum dapple viroid, ASSV: apple scar skin viroid.

unit plus, and one, two and four unit minus strands were not infectious. From these data, Ishikawa et al.[4] proposed a revised rolling circle model for viroid replication. Thus, HSV replication is thought to involve a site-specific cleavage of plus strand RNA multimers produced by a rolling circle mechanism to yield unit length molecules[12].

Momma and Takahashi[13,14] have described the cytopathic changes in the symptomatic leaves of hops or cucumbers infected with HSV. The most common changes incited by the viroid were the distorted cell wall and disorganized chloroplast. The same results were also obtained in citrus exocortis viroid (CEV)-infected terminal leaves of tomatoes that develop severe symptoms including leaf epinasty and/or rugosity[10,42]. Evidence for the absence of any ultrastructural changes in the nuclei strengthen the hypothesis that HSV or CEV is presumably replicated in nuclei without any conspicuous effects on nuclear structure examined so far.

From the analyses on endogenous indoleacetic acid (IAA) and gibberellin (GA_3), it was noted that IAA content was consistently lower in HSV-infected cucumbers compared with uninfected ones, but GA_3 levels were unaffected by the infection[52]. Significant lower levels of IAA observed in infected plants correlated well with leaf rugosity and delayed rate of female flower formation. If the decrease in number of female flowers in infected cucumbers (monoecious plant) would be controlled by the endogenous IAA levels, the corresponding mechanisms would occur in the case of HSV-infected hops (dioecious plant). The assumption was mainly based on the data of Yamamoto et al.[57] who compared the numbers of cones (female flowers) developed on severely affected hops and found out 24–58% of those of HSV-free hops.

Symptoms and transmission of viroids

In viroid diseases, characteristic stunting of the entire plants is known to be common and there are usually other symptoms such as smaller upper leaves, and internodal shortening in plants infected with HSV or CEV. Severely affected hop plants with HSV showed downcurling and/or rugosity over the whole length of the leaf, and their cones were also small and more elongated than normal[37,57]. Discoloration of the leaves such as mosaic or mottling has scarcely observed in any viroid-infected original hosts.

On the other hand, woody plants infected with viroids showed different responses. For example, citrus trees infected with CEV were severely stunted and exhibited bark shelling on their rootstocks[53]. Scar skin and/or dapple apple symptoms after infection with apple scar skin viroid (ASSV) developed only on the apple fruits, leaving the foliage and trunk of the affected trees normal appearance[55]. These symptoms differ among the apple varieties tested. A peculiar dapple symptom has also been observed on plum fruit infected with plum dapple viroid[27,46]. The summary of symptoms developed on the original hosts is

Table 2. Viroid symptoms developed on original hosts, and indicator plants for indexing viroid currently used in Japan

Viroid*	Original host	External symptoms on original host	Indicator plant
CEV	Citrus	Bark shelling of rootstocks and stunting of the tree	Ethrog citron, *Gynura aurantiaca*, Rutgers tomato
HSV	Hop	Shortened internodes of the main and lateral bines and curling of upper leaves	Suuyou cucumber
CSV	Chrysanthemum	Stunting of the plant and smaller leaves	Mistletoe chrysanthemum
ASSV	Apple	Scar skin or dapple symptom on fruit	—
PDV	Plum	Dapple symptom on fruit	—

* See the footnotes of Table 1.

shown in Table 2.

All viroids found in Japan are easily transmissible by grafting. Some viroids are often transmitted in the field by contaminated tools and cultivating equipments. For example, CEV can be mechanically transmitted with contaminated budding knives, even though in has been difficult to transmit by sap inoculation of citrus leaves. In the case of HSV[57], transmission in the hop garden has also occurred when contaminated sickles or bare hands were used to dress or pull bines of the hop plants. The disease spreads often along the ridges of the gardens. Chrysanthemum stunt viroid was shown to be transmitted during the time of cultural operations using contaminated knives, tools or bare hands. Since mechanical transmission of ASSV to apple seedlings by razor-slashing has been demonstrated, it was suggested to indicate that causal agents of the disease can be transmitted by pruning operations in the orchard[8].

Tests for seed transmission of HSV indicated that neither pollen transmission nor ovule transmission occurred in hop or tomato plants[51]. Also negative transmission results for HSV have been reported with some vectors such as nematodes or green peach aphid, *Myzus persicae*[57]. No vectors have yet been elucidated so far. For other four viroid diseases occurring in Japan, no transmission through seed, soil and/or through the insect could be demonstrated.

Indexings for viroid diseases

The original hosts of several viroids found in Japan have long incubation periods of 1–3 years after inoculation. This is one of the most serious disadvantages for the diagnosis of viroid diseases. Addition to the symptom diagnosis of original hosts, there are three methods for detecting viroids; 1) bioassay, 2) PAGE analysis and 3) nucleic acid hybridization test.

Except for apple and plum viroids, some viroids can be tested with indicator plants (Table 2). With tests for susceptibility to HSV infection, 12 out of 26 cucumber cultivars become infected severely, so that visual symptoms are reliable for diagnosis because symptom severity is correlated with viroid content[50]. The infectivity assay of purified HSV using cucumber indicated that dilution end point for infection was 10–100 pg/ml[58]. In bioassay, high temperatures have been often used to develop characteristic symptoms and aid in indexing viroid diseases. For example, when cucumber plants for HSV indexing were placed at 32°C by day and 27°C by night or at day and night temperature of constant 32°C, the incubation period was usually 14 days. At 25°C, however, it was found that, each of the inoculated plants did not develop any distinct symptoms at 30 days after inoculation, but all of them were infected inapparently, as judged from back-inoculation[38]. Similar response to high temperature has also been observed in CEV-inoculated *Gynura aurantiaca* or tomato plants[6]. Tomato bioassay for CEV has already been established[16].

All viroids can be tested by the PAGE analysis. Various modifications of PAGE method have been successfully developed and enable the entire tests to be completed within several hours[10]. In parallel with this, we have used a nucleic acid hybridization test to detect HSV in infected hop leaves[36]. Our studies have thus confirmed that the nucleic acid hybridization test to be more sensitive than the cucumber bioassay or the PAGE anlaysis, as has been observed for other viroids[36]. By using a technique of nucleic acid hybridization, a viroid molecule (GV) carried symptomlessly in cultivated grapevine was found[25,33], and its complete nucleotide sequence was established[26]. It consists of 297 nucleotides and differs in one nucleotide from the sequence of HSV. GV was also isolated from grapevines recently introduced into Japan from Austria, France, Hungary, West Germany and U.S.A.[28].

Control measures of viroid diseases

No chemotherapeutants are yet available for controlling the viroid diseases. Therefore,

control measures have to correspond to the breaking of the infection chains for each viroid. From the epidemiological studies on natural infection with several viroids, practical control measures suggested themselves. They are 1) prompt removal of all the infected plants including root systems, 2) replanting with certified viroid-free plants and 3) disinfection of the tools contaminated with viroids.

First of all, the most satisfactory procedure of disease eradication was the removal of diseased plant tissues and their residues. From the survey on HSV distribution, HSV cannot survive in any weeds or wild plants in the severely infested hop garden[51]. Although infectivity of HSV was relatively stable, hop plant residues, leaves and cones, were decayed within three months when left to be weather-beaten, thus leading to loss of HSV infectivity[51]. However, HSV can persist in the infected root systems in the hop garden for years. This finding emphasizes the need for complete roguing of the infected root systems or for careful treatments with herbicides, such as picloram or glyphosate, to destroy the root systems as well as to inactivate HSV infectivity (Takahashi, T., unpublished data).

Since vegetatively derived cuttings (or scions) of viroid-infected plants are undoubtedly infected, such cuttings used for establishing planting material become a potent source of the viroid. To obtain the certified plants free of the viroid, it is most important to distribute viroid-free cuttings or viroid-free shoot apical meristems, which were taken from viroid-free stocks or meristem tip cultures, after inspection by the several indexings. In the infected hop plants, HSV concentrations tested by cucumber bioassay are gradually decreasing towards the shoot tips, and the upper 0.2 mm of the shoots appears to be practically HSV-free. Thus, HSV can be quite successfully eliminated from shoot apical meristems comprising the apical dome plus the first two primordia from which viroid-free plants are grown[14]. By using a combination of low temperature treatment of the infected plants and subsequent meristem tip culture, low temperature ($10 \pm 2°C$ and 4,400 to 6,400 lux 16 hr/day for 1, 2, 3 or 4 months) did not enhance the efficiency for production of HSV-free hop plants[14].

As has been mentioned above, several viroids are mechanically transmitted by tool, hands and other direct contact with the healthy plants. Therefore, precaution against contact transmission is absolutely essential. In some instances, HSV-contaminated sickles and/or knives, which had been kept under dried condition for one year, were shown to become serious sources of HSV infection. With search for disinfectants, recommended chemicals for tools contaminated with HSV were 1% formaldehyde, 1% sodium hydroxide, 5% sodium hypochlorite, 5% calcium hypochlorite and 5% trisodium phosphate[39]. A solution of 2% formaldehyde plus 2% sodium hydroxide was also found useful in preventing HSV transmission[39]. Ushiyama[48] has already demonstrated the effectiveness of sodium hypochlorite or a solution of formaldehyde plus sodium hydroxide for disinfecting tools for control of CEV. Infectivity of HSV was highly tolerant to thermal inactivation even in the LiCl-soluble fraction. The exposure of the contaminated razor blades to heat for 10 min at 140°C did not completely prevent transmission of HSV[39]. Thus, we recommended that heating tools for 30 min at 180°C was practically effective as the procedure used for disinfecting tools carrying HSV[39].

Conclusion

In viroid diseases, several environmental factors affect on natural occurrence, symptom production and transmission. One may conclude that perennial plants and/or vegetatively propagated plants are occasionally susceptible to viroids and become potent viroid-carriers for a long time. These plants thus infected may pose a serious threat to successful crop production. Fortunately, there are no insect vectors of viroid diseases found in Japan, so diseased plants should be de-

tected and removed, and they are not permitted to perpetuate the viroid reservoirs through successive cultivation.

Recently, it was evident that there exists some viroid isolates that inapparently infect to plants such as grapevine isolate of HSV in grapevine or citrus isolate of HSV in citrus. However, there have yet been no reports of significant loss in respective plants. This suggests to indicate that viroid can adapt to some host plants and to wide range of the climates that impose the restriction on viroid survival in nature.

Plant pathologists in Japan have faced many troubles such as unknown etiology of viral syndrome, viroid complexes associated with citrus to which several viroid species to infect inapparently, accidental transmission of viroids in the fields, breeding of transgenic plants transformed with the attenuated viroid molecule, strategies for urgent diagnosis of viroid diseases. Some of these troubles can be solved to reach a final aim in several viroid diseases. However, in our opinion there are still possible solutions towards further viroid research.

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