Hop Stunt Viroid Strains from Dapple Fruit Disease of Plum and Peach in Japan

By TERUO SANO, 1* TATSUJI HATAYA, 1 YASUO TERAI 2 AND EISIHO SHIKATA 1

1*Department of Botany, Faculty of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo 060 and 2 Yamanashi Fruit Tree Experiment Station, Yamanashi 405, Japan

(Accepted 21 February 1989)

SUMMARY

Viroids have been isolated from plum trees (Prunus salicina Lindley) affected with plum dapple fruit disease and from peach trees (Prunus persica Batsch) showing dapple fruit symptoms. The viroids were inoculated mechanically to cucurbitaceous plants, in which symptoms typical of hop stunt viroid (HSV) infection appeared. The complete nucleotide sequences of an isolate from plum and an isolate from peach (AF isolate) were shown to be identical, consisting of 297 nucleotides with a 93.6% sequence homology to HSV-hop. Another isolate from peach (A9 isolate) also consists of 297 nucleotides, but the sequence homology to HSV-hop is 99.7%, showing only one nucleotide replacement. These results indicate that these three viroids are strains of HSV, which we designate HSV-plum, HSV-peach (AF) and HSV-peach (A9), respectively. Comparative analysis of the nucleotide sequences of HSV strains from hop, grapevine, citrus, cucumber, plum and peach revealed variable and conserved regions in the HSV molecule. In Japan, these viroids are closely related not only to dapple fruit disease in plum cv. Taiyo, but also to dapple fruit symptoms on peach cv. Asama-Hakutou.

INTRODUCTION

A newly recognized disease of plum (Prunus salicina), characterized by red blotches on fruits of cvs. Taiyo and Oishi-Wase Sumomo, was detected in Yamanashi Prefecture, Japan (Terai, 1985). The disease is transmitted by grafting and was named plum dapple fruit disease (Terai, 1985). Another disease of plum, characterized by a yellowish red colour in the fruit flesh of cv. Soldam, was also reported in the same prefecture and was named Soldam yellow fruit disease (Terai, 1987). Both diseases seem to be caused by the same pathogen, as healthy plum trees cv. Taiyo which were graft-inoculated with cv. Soldam showing yellow fruit symptoms developed dapple fruits, and vice versa (Terai, 1987). A similar symptom, consisting of chlorotic blotches on the fruit of peach (Prunus persica) cv. Asama-Hakutou has also been recognized in Yamanashi Prefecture (Y. Terai, unpublished observations).

A low Mr RNA of viroid-like nature, associated with dapple fruits of cv. Taiyo, has an Mr, similar to that of hop stunt viroid (HSV)-grapevine and has a relatively high sequence homology with the HSV group (Sano et al., 1986b).

In this report, we characterize the viroids isolated from plum and peach dapple fruits. Our results show that the viroids are new members of the HSV group and that they are closely related to dapple fruit disease on plum and peach. In addition, variations in the nucleotide sequences within the HSV group, isolates of which have world-wide distribution in orchard plants, are discussed.

METHODS

Plum and peach sources. Fruits and leaves of plum trees (P. salicina Lindley cv. Taiyo) affected with dapple fruit disease (Terai, 1985) were collected from Yamanashi Prefecture, Japan. Fruits and leaves of peach trees (P. persica Batsch cv. Asama-Hakutou) were also collected from Yamanashi Prefecture. An isolate designated DF-peach
AF, showing severe dapple fruit symptoms, and an isolate designated DF-peach A9, showing mild dapple fruit symptoms, were used.

**Purification and PAGE.** Low Mr RNA molecules were extracted from frozen leaves, buds, fruit skin and flowers by using the method of Uyeda et al. (1984) with a slight modification. Frozen tissues were homogenized in 130 mM-Tris-HCl pH 8.9, 17 mM-EDTA pH 7.0, 0.83% SDS, 1 M-LiCl, 5% polyvinylpyrrolidone and 1% mercaptoethanol (Flores et al., 1985), instead of 1 M-K2HPO4. After phenol extraction, 2-methoxyethanol extraction, cetyltrimethylammonium bromide precipitation, 2 M-LiCl fractionation and DNase I digestion, the RNA molecules were absorbed to CF-11 cellulose in a buffer containing 35% ethanol, washed with the same buffer and then eluted with ethanol-free buffer.

The extracted RNA molecules were analysed by a two-dimensional (2D) electrophoretic technique followed by silver staining (Schumacher et al., 1983), 15% PAGE under non-denaturing condition (Loening, 1967) and 5% PAGE containing 8 M-urea under denaturing conditions (Sänger et al., 1979).

Further purification was achieved by 15% PAGE under non-denaturing condition. Viroid bands were excised from the gel and viroid RNA was recovered by electro-elution (ISCO concentrator), precipitated by ethanol, redissolved in the original volume of distilled water and stored at -30 °C.

**Infectivity assay on herbaceous test plants and host range.** Low Mr RNAs extracted from plum and peach were rubbed-inoculated onto cucumber (Cucumis sativus L. cv. Siyō). Then low Mr RNAs were extracted from cucumber which showed symptoms and inoculated onto 16 species of Cucurbitaceae, five species of Solanaceae and two species of Compositae to test the host range of viroids that produced disease symptoms on cucumber. After inoculation, plants were kept for 6 weeks in a glasshouse at 25 °C to 32 °C, with a day length of 16 h. Plants showing no symptoms were each back-inoculated to four cucumber plants to check whether the plants had been latently infected.

**Dot blot hybridization.** Low Mr RNAs were spotted onto a nitrocellulose filter described previously (Sano et al., 1988b) and hybridized with an 32P-labelled oligonucleotide probe (probe HSV-1, 5' GTTGCCC CCGGCGTCTCCT 3', common to the HSV group; Yuki Gosei Kogyou Company Ltd). Hybridization was performed at 55 °C (Sano et al., 1988b).

**Cloning and sequencing.** Viroid RNA purified from infected cucumber plants was annealed with an oligonucleotide primer (5' GGTAAGTACCTCCCT 3'; Yuki Gosei Kogyou Company Ltd), and first strand cDNA and second strand ds cDNA were synthesized by the method of Gubler & Hoffman (1983). After dC-tailing, the cDNA was cloned into PstI-cleaved, dG-tailed pBR322 plasmids. Escherichia coli (HB101) transformants containing cDNA inserts were screened, the cDNA fragments were excised from the plasmid vector by restriction endonucleases and were subcloned into M 13 mp 19 for sequencing by the dideoxynucleotide method (Messing, 1983). The sequence determined by this method was confirmed by reverse transcriptase-dideoxynucleotide sequencing. For this analysis, purified viroid RNA was annealed with 5' end 32P-labelled oligonucleotide primers or cDNA primers prepared from an HSV cDNA clone, and reverse-transcribed as described previously (Sano et al., 1985).

**RESULTS**

**Infectivity assay**

Low Mr RNAs (100 µg/ml) extracted from healthy plum (cv. Taiyo), plum dapple fruits (cv. Taiyo, DF-plum), healthy peach (cv. Asama-Hakutou), peach mild dapple fruits (cv. Asama-Hakutou, DF-peach A9) and peach dapple fruits (cv. Asama-Hakutou, DF-peach AF) were rubbed-inoculated onto cucumber plants. In plants inoculated with DF-plum, DF-peach A9 and DF-peach AF, symptoms appeared 17 to 25 days after inoculation, and consisted of stunting, leaf curling and vein clearing (Table 1). The symptoms were similar to those induced by the HSV group viroids (Sano et al., 1986c).

To examine the host range of the viroids, low Mr RNA extracted from cucumber infected with DF-plum was inoculated onto 23 species of Cucurbitaceae, Solanaceae and Compositae. The symptoms appeared on Benincasa hispida, Cucumis melo, C. sativus, Lagenaria siceraria, Luffa cylindrica and Monardica charantia. Latent infection occurred on Citrullus vulgaris, Cucurbita moschata and Cucurbita maxima. The viroid did not infect Cucurbita pepo or Sechium edule in the Cucurbitaceae, nor did it infect the seven Solanaceae species or two species in the Compositae.

**Detection of a viroid-like RNA by 2D electrophoresis**

Low Mr RNAs (100 µg) were extracted from healthy plum, DF-plum, healthy peach and DF-peach AF and examined by 2D electrophoresis. A band which seemed to be a viroid-like circular RNA molecule was detected from both DF-plum (Fig. 1b) and DF-peach AF (Fig. 1d). No
Plum and peach HSV viroid strains

Fig. 1. 2D electrophoresis of RNA molecules extracted from healthy plum (a), DF-plum (b), healthy peach (c) and DF-peach AF (d). Arrows indicate the direction of electrophoresis.

Table 1. Cucumber assay

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Symptoms</th>
<th>Cucumber assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv. Taiyo</td>
<td>Dapple fruit</td>
<td>10/10*</td>
</tr>
<tr>
<td>cv. Taiyo</td>
<td>None</td>
<td>0/10</td>
</tr>
<tr>
<td>Peach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cv. Asama-Hakutou AF</td>
<td>Dapple fruit</td>
<td>24/24</td>
</tr>
<tr>
<td>cv. Asama-Hakutou A9</td>
<td>Mild dapple fruit</td>
<td>10/10</td>
</tr>
<tr>
<td>cv. Asama-Hakutou</td>
<td>None</td>
<td>0/24</td>
</tr>
</tbody>
</table>

* Numbers of plants infected/inoculated.
Fig. 2. Co-electrophoresis of viroid-like RNAs from dapple fruit plum (DF-plum), peach AF (DF-peach) and HSV isolated from hop (HSV-h), grapevine (HSV-g), citrus (HSV-cit) and cucumber (HSV-c) in 5% polyacrylamide gel containing 8 M-urea under denaturing conditions. Samples in lanes 7, 8, 9 and 10 were extracted from plum and peach, and the others were from cucumber. Lanes 5, 8 and 11 represent specimens extracted from healthy cucumber, plum and peach, respectively. C represents, as a marker, the circular molecule of HSV-grapevine; L, the linear molecule of HSV-grapevine.

corresponding band could be detected from healthy plum and peach (Fig. 1 a, e). Furthermore, no additional viroid-like circular RNA band could be detected in any of the four preparations.

Co-electrophoresis of a viroid-like RNA from plum and peach with HSV strains

Low Mr RNAs (100 μg) extracted from healthy plum, DF-plum, healthy peach, DF-peach AF, and from cucumber infected with DF-plum, DF-peach AF, HSV-hop, HSV-grapevine, HSV-cucumber and HSV-citrus were electrophoresed on a 15% polyacrylamide gel under non-denaturing conditions. After staining with ethidium bromide, the segment of the gel corresponding to the position where viroids (ranging from hop latent viroid to citrus exocortis viroid) were electrophoresed, was cut out from each lane and electrophoresed further on a 5% gel containing 8 M-urea under denaturing conditions (Flores et al., 1985). A viroid-like RNA detected from both DF-plum and DF-peach AF, corresponding to the viroid-like circular RNAs in Fig. 1 migrated at almost the same rate as HSV-hop and HSV-grapevine, but slightly faster than HSV-cucumber and HSV-citrus (Fig. 2). The concentration of the viroid-like RNA in DF-plum was much higher than that in DF-peach AF. The viroid-like RNA was infectious to cucumber and produced symptoms typical of those of the HSV group.

Dot blot hybridization

The low Mr RNA samples, which were the same as those used in the comparative PAGE analysis shown in Fig. 2, were hybridized with a synthetic oligonucleotide probe, HSV-1. The
Plum and peach HSV viroid strains

<table>
<thead>
<tr>
<th>Viroid</th>
<th>Host</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) HSV-h</td>
<td>Cucumber</td>
<td>x1</td>
</tr>
<tr>
<td>(b) HSV-g</td>
<td>Cucumber</td>
<td>×5</td>
</tr>
<tr>
<td>(c) HSV-cit</td>
<td>Cucumber</td>
<td>×25</td>
</tr>
<tr>
<td>(d) HSV-c</td>
<td>Cucumber</td>
<td></td>
</tr>
<tr>
<td>(e)</td>
<td>Cucumber</td>
<td></td>
</tr>
<tr>
<td>(f) DF-plum</td>
<td>Cucumber</td>
<td></td>
</tr>
<tr>
<td>(g) DF-plum</td>
<td>Plum</td>
<td></td>
</tr>
<tr>
<td>(h)</td>
<td>Plum</td>
<td></td>
</tr>
<tr>
<td>(i) DF-peach AF</td>
<td>Cucumber</td>
<td></td>
</tr>
<tr>
<td>(j) DF-peach AF</td>
<td>Peach</td>
<td></td>
</tr>
<tr>
<td>(k)</td>
<td>Peach</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Autoradiograph of dot blot hybridization using synthetic oligonucleotide probe HSV-1, which is complementary to all the strains of the HSV group. Fivefold dilutions of low Mr RNAs from dapple fruit plum (DF-plum), dapple fruit peach AF (DF-peach), healthy cucumber, healthy plum, healthy peach and HSV isolated from hop (HSV-h), grapevine (HSV-g), citrus (HSV-cit), cucumber (HSV-c), starting with 10 μg/spot were spotted on the filter. Hybridization was performed at 55 °C.

probe hybridized well with the low Mr RNAs from cucumber infected with DF-plum and DF-peach AF as well as with those from cucumber infected with four viroids of the HSV group (Fig. 3). Moreover the probe hybridized with low Mr RNA extracted directly from leaves of DF-plum (Fig. 3), which showed a thick band of the viroid-like RNA as shown in Fig. 2, lane 7. However, no detectable hybridization could be observed with the low Mr RNA extracted directly from leaves of DF-peach AF (Fig. 3), which showed a thin band of a viroid-like RNA as shown in Fig. 2, lane 10. This result indicates that the viroid-like RNAs in DF-plum and DF-peach AF are viroids belonging to the HSV group, because the probe HSV-1 is specific for the HSV group (Sano et al., 1988b).

Comparison of the nucleotide sequences of viroids isolated from DF-plum and DF-peach AF and A9 with those of the HSV group

Nucleotide sequences of viroids isolated from DF-plum and DF-peach AF and A9 were established and compared with those of the known viroids (Table 2). All the sequences obtained form circular molecules consisting of 297 nucleotides, which can form rod-like structures with the extensive base pairing characteristic of viroids and have high sequence homology to the viroids in the HSV group. The nucleotide sequences of DF-peach AF and DF-plum are the same (Fig. 4). The sequence homology between these isolates and HSV-hop is high (93.6%) and the right-hand structures of these isolates and HSV-hop are identical. The differences between these isolates and HSV-hop are present in the left-hand portion of the molecules, including the lower portion of the central conserved region.
Fig. 4. Comparison of the nucleotide sequences of DF-plum, DF-peach A9 and DF-peach AF to those of the HSV group. Nucleotides which are different from those of HSV are indicated by arrows.


**Plum and peach HSV viroid strains**

Table 2. Distribution of and relationships among the HSV group

<table>
<thead>
<tr>
<th>Type</th>
<th>Viroid</th>
<th>Natural host</th>
<th>Disease symptoms</th>
<th>Countries</th>
<th>Nucleotides (no.)</th>
<th>Variations*</th>
<th>Homology among strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hop</td>
<td>HSV-h</td>
<td>Hop</td>
<td>Hop stunt</td>
<td>Japan</td>
<td>297</td>
<td>1 0 0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>HSV-peach (A9)</td>
<td>Peach</td>
<td>Unknown or dapple fruit</td>
<td>Japan</td>
<td>297</td>
<td>1 0 0</td>
<td>99-7</td>
</tr>
<tr>
<td></td>
<td>HSV-g</td>
<td>Grapevine</td>
<td>Unknown or latent</td>
<td>Japan, China, U.S.A., Austria, F.R.G., France, Spain, Australia, and Hungary</td>
<td>297</td>
<td>1 0 0</td>
<td>99-7</td>
</tr>
<tr>
<td>Plum</td>
<td>HSV-plum</td>
<td>Plum</td>
<td>Plum dapple fruit</td>
<td>Japan</td>
<td>297</td>
<td>13 3 3</td>
<td>93-6</td>
</tr>
<tr>
<td></td>
<td>HSV-peach (AF)</td>
<td>Peach</td>
<td>Soldam yellow fruit</td>
<td>Japan</td>
<td>297</td>
<td>13 3 3</td>
<td>93-6</td>
</tr>
<tr>
<td>Citrus</td>
<td>HSV-cit var. 2</td>
<td>Citrus</td>
<td>Unknown or latent</td>
<td>Japan</td>
<td>302</td>
<td>7 6 1</td>
<td>97-0</td>
</tr>
<tr>
<td></td>
<td>HSV-cit var. 1</td>
<td>Citrus</td>
<td>Unknown or latent</td>
<td>Japan</td>
<td>302</td>
<td>7 7 2</td>
<td>96-3</td>
</tr>
<tr>
<td></td>
<td>HSV-c</td>
<td>Cucumber</td>
<td>Cucumber pale fruit</td>
<td>Netherlands</td>
<td>303</td>
<td>8 7 1</td>
<td>96-3</td>
</tr>
</tbody>
</table>

* Sub, substitution; Ins, insertion; Del, deletion.

The sequence of DF-peach A9 causing mild dapple fruit symptoms is more closely related to that of HSV-hop than that of DF-peach AF (Fig. 4). The difference between HSV-hop and DF-peach A9 is only one nucleotide, at position 201 (99-7% sequence homology).

**DISCUSSION**

It was shown that both viroids isolated from plum and peach were closely related to HSV, on the basis of their pathogenicity to cucurbitaceous plants, analysis by PAGE, molecular hybridization and finally nucleotide sequence homology. Symptoms of these viroids on cucumber plants (cv. Súyô) were almost the same as those caused by HSV-hop, -grapevine, -cucumber and -citrus under the same glasshouse conditions. The host range of the viroid from DF-plum resembles that of HSV-hop and of HSV-cucumber reported previously (Van Dorst & Peters, 1974; Runia & Peters, 1980; Sano et al., 1981). A slight difference in host range was recognized on tomato plants. So far, the viroid from DF-plum has not been shown to infect tomato, but HSV-hop, -grapevine, -cucumber and -citrus infected tomato without showing visible symptoms.

Molecular hybridization and sequence analysis revealed that both viroids have high (more than 90%) sequence homology to HSV isolates. In particular, DF-peach A9 was different by only one and two nucleotides from HSV-hop and HSV-grapevine, respectively. Accordingly we identified these viroids as peach strains of HSV, designated HSV-peach (A9). These three isolates are closely related to each other, and seem to form one type (hop type). On the other hand, DF-plum and DF-peach AF, having the same sequence, are distantly related to the hop type of the HSV group. They are more closely related to an isolate of HSV from the German grapevine (cv. Riesling) (Puchta et al., 1988). The latter isolate is different at eight positions from HSV-hop, and seven out of those eight are the same nucleotides as we found in the DF-peach AF isolate. Although DF-peach AF is different in an additional 12 positions from HSV-hop, the isolate is identified as the plum strain of HSV, designated HSV-plum [or HSV-peach (AF)]. HSV-plum and HSV-grapevine (Riesling) seem to form the plum type of the HSV group. Furthermore, the isolates from cucumber and citrus reported previously (Sano et al., 1984, 1988a) are closely related to each other, and seem to form the citrus type of the HSV group.
Fig. 5. Variable and conserved regions in the HSV molecule. Arrowheads (▸) indicate the positions where variations in nucleotide sequence have been recognized among strains. Boxes (□) indicate the residues of HSV sequence common to those of potato spindle tuber viroid. Stars (★) indicate the positions of mutations that make HSV non-infectious, introduced by an in vitro mutagenesis technique shown by Ishikawa et al. (1985). 'C-Region' represents the conserved regions.

In recent years various isolates of HSV have been detected from various species of plants in many countries (Sano et al., 1986c; Puchta et al., 1988; Rezaian et al., 1988). They can be classified into three types as indicated above, i.e. the hop type, plum type and citrus type. Comparative analysis of their nucleotide sequences revealed the existence of conserved and variable regions in the HSV molecule. The upper portion of the central conserved region (bases 60 to 114 in HSV-hop), the left-hand end portion (267 to 24 in HSV-hop) and the right-hand portion of the HSV molecule are conserved (Fig. 5). The importance of the upper portion of the central conserved region (named region A) for HSV replication was indicated previously by analysis of the infectivity of deletion mutants of HSV derived from an infectious dimeric cDNA clone (Meshi et al., 1985). The conserved central upper portion observed in natural isolates of HSV is consistent with region A, which is believed to include the splicing junction used in viroid replication (Diener, 1986). In addition, the latter two portions indicated above will be also important to HSV replication, because three mutations induced in these part by an in vitro mutagenesis technique made HSV non-infectious (Ishikawa et al., 1985). On the other hand, variable regions are located at both sides of the central conserved region. The location of the one on the left side corresponds to the pathogenic modulating region in the potato spindle tuber viroid (Schnölder et al., 1985) and the citrus exocortis viroid (Keese & Symons, 1985), although all the HSV isolates produced quite similar symptoms on cucumber plants.

Based on the results of sap inoculation experiments and analysis by PAGE, it is most likely that HSV-plum causes dapple fruit disease on plum cv. Taiyo. In addition, on peach plants, HSV-peach (AF) (synonym for HSV-plum) or HSV-peach (A9) seem to be associated with the dapple fruit symptom. These two isolates have different nucleotide sequences: HSV-peach (AF) isolated from peach showing severe dapple fruit symptoms has the same nucleotide sequence as HSV-plum from typical dapple fruit plum, but HSV-peach (A9) isolated from peach showing mild dapple fruit symptoms has a different nucleotide sequence. But we should note the possibility that an unknown viroid or another pathogen that cannot be detected by our procedures (cucumber assay or PAGE) but which has an important role in disease expression, exists in plum and peach trees. To elucidate the aetiology of HSV-plum, HSV-peach (AF) (synonym for HSV-plum) and HSV-peach (A9), back-inoculations of the viroids to plum and peach trees are now under way, though it may take several years to confirm their pathogenicity.

Since HSV was detected from Japanese hop plants, a group of similar agents, named the HSV group, has been shown to be distributed in various species of orchard plants, such as hop, grapevine, citrus, peach and plum. Some of these strains of HSV cause serious diseases on hop, cucumber, plum and possibly peach, but seem to infect grapevine and citrus latently (Table 2). It should be noted that, as a high percentage of HSV infection without detectable symptoms has been reported in grapevine and citrus (Sano et al., 1986a,c) these plants would seem to have an important role in HSV epidemiology as a potential source of the viroid.
Plum and peach HSV viroid strains

We would like to thank Dr T. O. Diener for his critical reading of the manuscript. This work was supported in part by Grants-in-Aid for Scientific Research nos. 60760039, 61760044, 62760040 and 63760039, Grant-in-Aid for Developmental Scientific Research no. 60860006 from the Ministry of Education, Science and Culture of Japan, a Kuribayashi Grant, 1985 and an Inamori Grant, 1986. We thank The Research Center for Molecular Genetics, Hokkaido University for the use of the glasshouse.

REFERENCES


(Received 6 October 1988)