A Viroid-like RNA Isolated from Grapevine Has High Sequence Homology
with Hop Stunt Viroid

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SUMMARY

Viroid-like RNA was isolated from some samples of grapevine leaves (Vitis vinifera
L.). When inoculated to cucumber, this RNA induced symptoms that were identical to
those induced by hop stunt viroid (HSV). It had a molecular size similar to that of HSV,
and hybridized with a 32P-labelled DNA probe for the cucumber isolate of HSV (HSV-
c, pCP-55). This hybridization suggests high (> 55%) sequence homology with HSV.
The HSV-related sequence was detected not only in the infected cucumber plants but
also directly in RNA extracted from grapevine plants. The result indicates that viroid-
like RNA, closely related to HSV, is present in grapevines in Japan.

INTRODUCTION

Viroids are infectious low molecular weight RNA molecules that can cause serious diseases of
higher plants (Diener, 1979). So far, more than 10 viroids, including isolates, have been
sequenced and have been found to comprise 250 to 370 nucleotides (Sänger, 1982; Visvader &
Symons, 1983; Kiefer et al., 1983; Ohno et al., 1983; Sano et al., 1984). Among the viroids
reported from various countries in the world, hop stunt viroid (HSV), which has 297 nucleotides,
was endemic only to Japan (Yamamoto et al., 1970), although most commercial hops in Japan
were introduced from Europe or America. Sasaki & Shikata (1977a, b) showed that cucumber
pale fruit viroid (CPFV), reported only in Europe, was biologically identical to HSV. Recently,
we determined the sequence extending to 303 nucleotides and the secondary structure of CPFV
RNA, and found it to have 95% sequence homology with HSV (Ohno et al., 1983; Sano et al.,
1984). We therefore concluded that CPFV was a cucumber isolate of HSV (HSV-c).

In this paper, we report the finding of a viroid-like RNA in grapevines, which also has a high
sequence homology with HSV. This is the first report of a viroid-like RNA in grapevines.

METHODS

Sources of grapevines. Leaves of grapevine plants (Vitis vinifera L.), samples A, B, C, D (Table 1), were collected
in Yamanashi Prefecture and samples E, F, G, H were from Iwate Prefecture. Table 1 shows the virus symptoms
in the source plants. Samples A and B are different plants of the same variety. Sample C was from a meristem-
cultured plant derived from the plant which gave sample D and was free from fan leaf, leaf roll and fleck diseases
(Iri et al., 1982). Samples F and H were from plants not showing virus symptoms but which were not meristem-
cultured plants (Table 1).

Extraction of low molecular weight RNA. Low mol. wt. RNA was extracted from the grapevine leaves and is
hereafter called grapevine RNA-A, grapevine RNA-B, etc. as described previously (Uyeda et al., 1984). Extracts
from each grapevine sample or sap from cucumber infected with HSV and HSV-c were mechanically inoculated to
cucumber cotyledons (Cucumis sativus L. cv. Suyo'). Low mol. wt. RNA was extracted from these cucumber plants
to give cucumber RNA-C, RNA-D, RNA-HSV and RNA-HSV-c, respectively.

Polyacrylamide gel electrophoresis (PAGE). This was carried out in 15% gel in 0.04 M-Tris, 0.02 M-acetate, 0.001 M-
EDTA (Loening, 1967). After electrophoresis, gels were stained with ethidium bromide (0.5 µg/ml) and observed
using a u.v.-transilluminator.

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Table 1. Infection of cucumber plants by low mol. wt. RNA extracted from grapevines

<table>
<thead>
<tr>
<th>Sample</th>
<th>Grapevine cv.</th>
<th>Symptoms in vine</th>
<th>Infectivity for cucumber</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Cabernet Sauvignon</td>
<td>Virus-free*</td>
<td>0/6*</td>
</tr>
<tr>
<td>B</td>
<td>Cabernet Sauvignon</td>
<td>Leaf roll, fleck</td>
<td>6/6</td>
</tr>
<tr>
<td>C</td>
<td>Zenkoji</td>
<td>Virus-free*</td>
<td>0/18</td>
</tr>
<tr>
<td>D</td>
<td>Zenkoji</td>
<td>Leaf roll, fleck</td>
<td>13/13</td>
</tr>
<tr>
<td>E</td>
<td>Chardonne</td>
<td>Leaf roll</td>
<td>0/3</td>
</tr>
<tr>
<td>F</td>
<td>Alpha</td>
<td>Symptomless</td>
<td>3/4</td>
</tr>
<tr>
<td>G</td>
<td>Weiserblugndor</td>
<td>Leaf roll</td>
<td>4/4</td>
</tr>
<tr>
<td>H</td>
<td>Weiserblugndor</td>
<td>Symptomless</td>
<td>4/4</td>
</tr>
</tbody>
</table>

* Virus-free: viruses were eliminated by meristem culture (sample C) or heat therapy (sample A), and plants were shown to be free from leaf roll, fleck and fan leaf diseases.
† Number of cucumber plants infected/no. inoculated, 6 weeks after inoculation. Infected plants showed stunting, leaf curling and vein clearing.
‡ Symptomless: plants showed no symptoms, but virus had not been deliberately eliminated.

Northern blot hybridization. $^{32}$P-labelled DNA probe for HSV-c (pCP-55; Sano et al., 1984), with a specific activity of $2 \times 10^7$ c.p.m./pg DNA, was prepared by 5'-end labelling (Maniatis et al., 1982). The low mol. wt. RNA was glyoxalated and electrophoresed in 2% agarose gel (Carmichael & McMaster, 1980), transferred to nitrocellulose paper (Schleicher & Schuell) and hybridized with the $^{32}$P-labelled DNA probe for HSV-c ($5 \times 10^5$ c.p.m./ml) (Thomas, 1980). The nitrocellulose papers were then washed, dried and exposed to X-ray films at $-70^\circ$C. Fragments of pBR322 DNA formed by HpaII digestion were used as size markers.

RESULTS

Symptoms on cucumber plants inoculated with low mol. wt. RNA from grapevines

Samples of low mol. wt. RNA extracted from grapevines were inoculated to cucumber plants. Those inoculated with grapevine RNA-B, -D, -F, -G and -H became stunted, and showed leaf curling and vein clearing by 21 to 30 days after inoculation (Table 1). Each extraction induced similar symptoms (Fig. 1) which were identical to those induced by HSV. The cucumber plants inoculated with grapevine RNA-A, -C and -E showed no symptoms.

Detection of the specific RNA by PAGE

Grapevine RNA-C, -D, cucumber RNA-D, -HSV and -HSV-c were electrophoresed on a 15% gel (Fig. 2). Cucumber RNA-D (lane d) contained a specific band (fraction 5) which could not be detected in healthy cucumber RNA (lane c) and which migrated at the same rate as HSV and HSV-c (lanes a, b, arrow v). However, grapevine RNA-D contained at least three extra bands (lane f, fractions 1, 2 and 3) which could not be detected in grapevine RNA-C (lane e) but none of them migrated like the specific band in cucumber RNA-D (arrow v). Grapevine RNA-D did not appear to contain a band corresponding to the specific band of cucumber RNA-D or the viroid bands of HSV and HSV-c (lane a, b, arrow v).

Infectivity assays of gel fractions

After electrophoresis, gels of cucumber RNA-D and grapevine RNA-D (Fig. 2d, f) were sliced into seven fractions as indicated in Fig. 2. RNA was eluted by homogenizing the gel slices in 0.5 M-ammonium acetate, 0.01 M-magnesium acetate, 0.1% SDS (pH not adjusted). Eluted RNA was precipitated by ethanol, dried, dissolved in 0.1 M-Tris-HCl pH 7.5, 0.01 M-EDTA, 0.05% bentonite and inoculated to cucumber plants.

As shown in Table 2, most infectivity was associated with fraction 5 of either extract and thus corresponded with the specific band in cucumber RNA-D (Fig. 2, arrow v) but not with any band detected in grapevine RNA-D by the ethidium bromide staining method. There was no infectivity associated with the three extra bands in grapevine RNA-D.
Viroid-like RNA from grapevine

Fig. 1. Cucumber plants (cv. Sūyo) 6 weeks after inoculation with grapevine RNA-D (left), or no inoculation (right).

(a)  (b)  (c)  (d)  (e)  (f)

Fig. 2. Electrophoresis in 15% polyacrylamide gel of cucumber RNA-HSV (a), cucumber RNA-HSV-c (b), healthy cucumber RNA (c), cucumber RNA-D (d), grapevine RNA-C (e) and grapevine RNA-D (f). All samples were electrophoresed in the same gel. Arrow *v* indicates the position of HSV and HSV-c RNA. Gels were sectioned into seven slices as indicated.
Fig. 3. Northern blot hybridization analysis of cucumber RNA-HSV (a), healthy cucumber RNA (b), cucumber RNA-HSV-c (c), grapevine RNA-C (d), grapevine RNA-D (e) and cucumber RNA-D (f). About 20 μg RNA was electrophoresed in each lane. O indicates the origin of electrophoreses. The nucleotide numbers in co-electrophoresed glyoxalated DNA fragments of HpaII-digested pBR322 are shown on the right.

Table 2. Infectivity of gel fractions from sample D on cucumber plants

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Grapevine RNA-D*</td>
<td>0</td>
</tr>
<tr>
<td>Cucumber RNA-D × 1†</td>
<td>1</td>
</tr>
<tr>
<td>Cucumber RNA-D × 10†</td>
<td>0</td>
</tr>
</tbody>
</table>

* Low mol. wt. RNA extracted from grapevine sample D.
† Low mol. wt. RNA extracted from cucumber inoculated with grapevine sample D undiluted (× 1) or diluted 10-fold (× 10).
‡ Number of cucumber plants infected 8 weeks after inoculating four plants with each sample.

Cross-hybridization among the infectious RNA in grapevine extracts and cDNA of HSV-c

Samples of 20 μg of grapevine RNA-C, grapevine RNA-D, cucumber RNA-D, -HSV and -HSV-c were denatured and electrophoresed in 2% agarose in 0.1 M-sodium phosphate buffer pH 7.0. After electrophoresis, the RNA was transferred to nitrocellulose paper and hybridized with the 32P-labelled DNA probe for HSV-c. The probe hybridized with cucumber RNA-HSV and -HSV-c, and with both cucumber and grapevine RNA-D, but not with grapevine RNA-C or healthy cucumber RNA (Fig. 3). These results suggested that the infectious RNA species in grapevine plants were related to HSV.

In further tests, RNA eluted from gel fractions 1 to 6 of cucumber RNA-D (Fig. 2d), which were the same preparations used for infectivity assays in Table 2, were electrophoresed
Viroid-like RNA from grapevine

![Image](image.png)

Fig. 4. Northern blot hybridization analysis of RNA eluted from each of six fractions of the 15% polyacrylamide gel in (d) of Fig. 2. Lanes 1 to 6, RNA eluted from gel fractions 1 to 6; lane 7, cucumber RNA-D. O indicates the origin of electrophoresis. Markers are as in Fig. 3.

separately in a 2% neutral agarose gel, blotted and hybridized as above. As shown in Fig. 4, the probe hybridized only with RNA from fraction 5.

The molecular size of the RNA that hybridized with cDNA to HSV-c was about 300 nucleotides. Fig. 3 shows that some species of RNA about 600 nucleotides and larger were detected in addition to the major component of 300 nucleotides.

**DISCUSSION**

We have extracted low molecular weight RNA from eight different grapevines and five of these preparations, when inoculated to cucumber, induced symptoms identical to those induced by HSV.

PAGE revealed that a specific band in cucumber RNA-D migrated at the same rate as HSV. Furthermore, infectivity was associated with this band or, in grapevine extracts, with presumptive RNA having the mobility of this band but not detected, probably because of its low concentration in the grapevine plants used.

Northern blot hybridization analysis demonstrated sequence homology between the infectious RNA from grapevine and HSV-c, and that this infectious RNA can be detected using a cDNA probe for HSV-c. In previous work (Sano et al., 1984), we found that, under the same hybridization conditions as used in this work, a DNA probe for HSV could detect HSV-c but not potato spindle tuber viroid, which have 95% and 55% sequence homology to HSV respectively. Accordingly, we consider the sequence homology between the infectious RNA of grapevine and HSV is high, because it is more than 55%. We therefore conclude that this RNA is a viroid because (i) it is infectious, (ii) its molecular size is about 300 nucleotides and (iii) it hybridizes with a DNA probe for HSV-c.

The RNA species of about 600 nucleotides and larger detected by Northern blot hybridization in grapevine and cucumber RNA-D in Fig. 3 may correspond to the longer than unit length RNA species reported for HSV and other viroids (Ishikawa et al., 1984).
We cannot be sure that the infectious RNA causes a particular disease, because only eight grapevine plants were examined in these experiments. Moreover, although four had leaf roll and/or fleck diseases, no infectious RNA was isolated in sample E infected with leaf roll disease and sample H, showing no visible symptom, contained infectious RNA. Further work is needed to survey the occurrence of the infectious RNA in grapevines and to assess its disease potential. This and experiments involving inoculation of infectious RNA into indicator plants for grapevine viruses have been initiated but may take 2 to 3 years to complete.

Because the infectious RNA was not found in the virus-free grapevine sample C, which was derived from sample D, it may be possible to eliminate the infectious RNA from grapevine by meristem culture.

As well as the infectious RNA, three other low molecular weight RNA species were detected in the grapevine affected with leaf roll and fleck diseases. These RNAs did not infect cucumber plants and their nature and roles are unknown.

As far as we are aware, this is the first report of a viroid-like RNA in grapevine.

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REFERENCES


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