Viroid-like Properties of an RNA Species Associated with the Sunblotch Disease of Avocados

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SUMMARY

A low mol. wt. RNA species is associated with sunblotch disease of avocados. The RNA (SB-RNA) was recovered from leaves and bark of symptomless carrier trees and from bark lesions of symptom-bearing trees. It was not detected in leaves or bark from lesion-free areas of symptom-bearing trees or in leaf or bark from healthy trees.

SB-RNA is soluble in LiCl and migrates in 20% polyacrylamide gels. In high salt buffers, it is resistant to ribonuclease A at a concentration of 1 μg/ml and is degraded only slowly at 10 μg/ml. The RNA is less resistant to ribonuclease A at low salt concentrations (0.01 M). The mobility of the SB-RNA in polyacrylamide gels is not affected by heat denaturation. The apparent mol. wt. of native SB-RNA is 65000 whilst under denaturing conditions it is 112000 to 115000.

SB-RNA occurs in high concentration in leaves of symptomless carrier trees being detected in 1 g of fresh leaf. In tissue fractionation experiments, SB-RNA was associated mainly with the chloroplast and endoplasmic reticulum fractions. It is concluded from these properties of SB-RNA that sunblotch is a viroid disease.

INTRODUCTION

Sunblotch is a major disease of avocado (Horne & Parker, 1931) which is found in many of the avocado producing countries (Thomas & Mohamed, 1979). The disease occurs in two forms (Wallace & Drake, 1962): (1) trees showing characteristic fruit and bark symptoms. Only a small percentage (2 to 5%) of fruits from such trees show symptoms, and only those few fruits with symptoms have seeds that carry the disease; (2) trees that are symptomless carriers. These show no fruit or bark symptoms themselves but symptoms will develop in healthy avocado scions grafted on to them. Most (86 to 100%) of the fruits from these symptomless carrier trees carry the disease and although seedlings from these remain symptomless, healthy scions grafted on to them may develop symptoms.

The disease agent is graft transmissible and the main mode of spread is through the use of undetected symptomless carriers as rootstocks. The causal agent of sunblotch has been thought to be a virus but direct evidence of this has not been forthcoming. Recently, we demonstrated the presence of a low mol. wt. RNA species in leaves of symptomless carrier trees and in bark lesions of infected trees and its absence from healthy trees (Thomas & Mohamed, 1979). Dale & Allen (1979) also reported the presence of a low mol. wt. RNA in avocados affected by sunblotch disease in Australia. This RNA species was shown to have some viroid-like properties and in this paper we present further evidence of the viroid-like nature of sunblotch RNA.
METHODS

Source of infected material. Seeds from an avocado tree (cv. Topa Topa) known to be a symptomless carrier of sunblotch disease were obtained from Dr J. M. Wallace, University of California, Riverside. Two trees, raised from these seeds in a glasshouse, were used as a source of infected leaves. These trees were confirmed as symptomless carriers by grafting healthy avocado scions on to them; the healthy scions developed symptoms of sunblotch 9 to 18 months later. Leaves from a known healthy source, avocado trees of the cv. Hass, were used as healthy controls. In subsequent experiments, leaves from other healthy trees were also used as controls. All the healthy trees were shown to be disease free by the grafting technique routinely used in indexing for sunblotch (J. M. Wallace, personal communication). Further confirmatory work was carried out on leaf material from a symptomless carrier tree obtained from Dr Da Graca, Citrus and Subtropical Research Institute, Nelspruit, South Africa.

Purification of nucleic acids. Initial extraction of nucleic acids from avocado leaves was carried out by the direct phenol method of Diener et al. (1977). However, the high levels of tannins and phenolic compounds present in avocado leaves caused subsequent problems during polyacrylamide gel electrophoresis (PAGE). We therefore used an adaptation of the method of Morris & Wright (1975), in which polyvinyl pyrrolidone (PVP-40) was included in the extraction medium to absorb tannins, and were able to obtain preparations with spectra typical of pure nucleic acids. The method was as follows: (1) 30 to 40 g of leaves were homogenized with 100 ml of GPS buffer (0.4 M-glycine, 0.2 M-Na2HPO4, 1.2 M-NaCl, 0.2 M-Na2SO3, 0.1 % sodium diethyldithiocarbamate, pH 9.5, with NaOH) plus 5 g SDS, 5 g PVP-40, 25 ml chloroform and 25 ml n-butanol at high speed in a Waring blender for 5 min. The homogenate was then centrifuged at 10000 g for 20 min. (2) The aqueous supernatant was collected and 25 ml of chloroform and 25 ml of 90 % phenol were added. The mixture was shaken vigorously at 25 °C for 20 min and then centrifuged at 10000 g for 20 min. (3) Step 2 was repeated three times. (4) After the third chloroform–phenol extraction, 3 vol. of cold, absolute ethanol were added to the aqueous phase and the nucleic acids precipitated overnight at –20 °C. (5) The nucleic acids were collected by centrifugation at 10000 g for 20 min, dried under vacuum and dissolved in 5 to 10 ml of sterile distilled water. (6) The solution was dialysed for 48 h against several changes of distilled water, and the nucleic acids were then precipitated by the addition of 3 vol. of cold ethanol and a few drops of 2 M-sodium acetate. (7) The precipitate was left overnight at –20 °C and the nucleic acid collected by centrifugation at 8000 rev/min for 10 min. The precipitate was washed with ethanol and then with ether and dried under vacuum. (8) The dried precipitate was dissolved in resuspension (RS) buffer containing 0.04 M-tris acetate, pH 7.5, 0.02 M-sodium acetate, 0.002 M-EDTA, 0.2 % SDS and 5 % sucrose for electrophoresis, or in 0.15 M-NaCl–0.015 M-Na acetate (SSC) buffer, pH 7.0, or in sterile distilled water.

Electrophoresis. PAGE was usually carried out in 2.5 % polyacrylamide–0.5 % agarose gels in a slab-gel apparatus (Reid & Bieleski, 1968) using the tris–phosphate–SDS buffer system of Loening (1968). Gels were prepared using an acrylamide:bisacrylamide ratio of 19:1, containing 0.1 % TEMED and 0.1 % ammonium persulphate. Nucleic acid samples dissolved in RS buffer were layered directly on to gels; when samples were dissolved in SSC buffer or water, glycerol was added to 5 % before layering on to gels. E. coli RNAs were used as markers (Bishop et al. 1967): 23S (mol. wt. 1.08 × 106), 16S (0.56 × 106), 5S (3.6 × 104) and 4S (2.6 × 104). Gels were electrophoresed for 2 to 3 h at 4 W (constant power), stained with 0.2 % toluidine blue in methanol:acetic acid:water (15:5:30) for 30 to 60 min at 37 °C and destained in 5 % acetic acid overnight at 25 °C. Higher concentration gels (4
and 5%) were prepared without agarose. Twenty per cent gels containing 2.5% bisacrylamide were prepared in 8 x 0.5 cm glass tubes.

Electrophoresis of nucleic acids under denaturing conditions was carried out in 2.5% polyacrylamide gels using Loening's tris-phosphate buffer containing 1.1 M-formalin. Nucleic acids in SSC buffer were denatured by adding formalin to 1.1 M and heating at 63 °C for 15 min (Boedtker, 1971); E. coli RNAs used as mol. wt. standards were treated in the same way.

Electrophoresis was also carried out under denaturing conditions using the urea buffer system (Reijnders et al. 1973). Nucleic acid samples were denatured in 10 M-urea-0.2% SDS in Reijnder's buffer at 60 °C and layered on to 2.5% polyacrylamide gels. Electrophoresis was carried out at 60 °C using buffer containing 8 M-urea, 0.02 M-tris-HCl and 2 mM-EDTA, pH 7.5.

Fractionation of subcellular components. In an initial experiment, avocado leaves were homogenized in low molarity buffer (1/10 GPS) with a Virtis homogenizer at 20,000 rev/min. The slurry was then centrifuged at 10,000 g max for 10 min; the supernatant was centrifuged for 2 h at 100,000 g in a Spinco 40 rotor. Nucleic acids were then extracted from the 10,000 g pellet, 100,000 g pellet and supernatant as described above.

Further experiments were carried out to separate subcellular components into 'nuclear', 'chloroplast', 'mitochondrial' and 'cytoplasmic' fractions. Fresh leaves (5 g) were homogenized in 10 ml sucrose medium (0.005 M-sodium phosphate, pH 7.2, 0.005 M-mercaptoethanol, 0.01 M-MgCl₂ and 0.5 M-sucrose; Zaitlin & Hariharasubramanian, 1972) using a chilled mortar and pestle. The resulting slurry was filtered through cheese cloth and centrifuged at 1000 g max for 10 min. The 1000 g pellet was resuspended by gentle agitation in sucrose medium and Triton X-100 was added to 1% to solubilize membranes. The solution was then centrifuged at 2000 g for 10 min to give the 'nuclear' (pellet) and 'chloroplast' (supernatant) fractions. The supernatant was then centrifuged at 20,000 g for 20 min to give the 'mitochondrial' (pellet) and 'cytoplasmic' (supernatant) fractions. GPS buffer was added to the fractions and nucleic acids were extracted as described above.

Chemicals. Ribonuclease A (5 x crystallized) was obtained from Sigma Chemical Co., St Louis, Miss., and a stock solution (1 mg/ml) was prepared in distilled water and boiled for 5 min at 100 °C to remove any traces of deoxyribonuclease. Chrysanthemum stunt viroid (CSV) used as a comparison in some gels, was obtained from Peter Palukaitis of the University of Adelaide, South Australia.

RESULTS

RNA species detected in avocado leaves and bark tissues

When total leaf nucleic acids from healthy, symptom-bearing and symptomless carrier trees were fractionated by PAGE, DNA, ribosomal RNA (23S, 18S, 16S), 4S and 5S RNA were detected (Fig. 1) in all samples. An additional RNA (SB-RNA), of mobility approx. 8S, was detected in nucleic acid preparations from leaves of symptomless carrier trees (Fig. 1a). It was not found in nucleic acid preparations from healthy plants (Fig. 1b) or from leaves of infected symptom-bearing trees. This SB-RNA migrated as a sharp band in 2.5%, 4% and 20% gels. Occasionally, another RNA species with a mobility of approx. 12S in gels was detected in RNA preparations from both healthy and symptomless carrier trees. This non-specific RNA was not soluble in 2 M-LiCl and was digested by low levels of ribonuclease A in SSC buffer, showing it to be a single-stranded (ss) RNA probably of plant origin.

SB-RNA appeared to be present in high concentration in leaves from symptomless
Fig. 1. PAGE of total nucleic acids from leaves of (a) sunblotch infected symptomless carrier avocado trees and (b) healthy trees. Electrophoresis was in 2.5% polyacrylamide gels using Loening's buffer system (Loening, 1968) at 4 W for 3 h. Note the presence of an extra nucleic acid band (SB-RNA) in nucleic acids from symptomless carrier leaves.
carrier trees, being readily detected by PAGE of nucleic acid extracts from as little as 1 g fresh leaf tissue. It was not detected in leaves from infected trees even when nucleic acids extracted from over 50 g of leaf tissue were examined.

As the symptoms of sunblotch in infected symptom-bearing trees are localized in the bark tissues of stems, we decided to investigate the possibility that the causal agent was confined to these lesions. Four sources of infected symptom-bearing trees were established by grafting scions from symptomless carrier trees on to healthy seedlings. Typical sunblotch symptoms (yellow-brown streaking of the bark of twigs and young branches) developed 9 to 18 months later. These lesions were cut out and the bark tissue was stripped from them. Nucleic acids were then extracted from the bark and analysed by PAGE. In addition to 25S and 18S ribosomal RNA and 5S and 4S RNA, a band corresponding to SB-RNA was detected in all samples tested from symptom-bearing trees. SN-RNA was also detected in bark of symptomless carrier trees but not in bark taken from lesion-free areas of symptom-
bearing trees or bark of healthy trees. It therefore appears that in infected symptom-bearing trees, SB-RNA was confined to the bark lesions on the stem and was not detected in bark or other tissues from the rest of the plant.

We estimated the relative concentration of SB-RNA present in leaves of various ages from a symptomless carrier tree by extracting nucleic acids from equal amounts of newly emerged leaves, fully developed leaves and senescing leaves. After electrophoresis on polyacrylamide gels, the relative concentrations in the three samples were estimated by a visual assessment of the intensity of the SB-RNA bands. The results showed that the highest concentration of SB-RNA was in mature leaves followed by young leaves, with barely detectable amounts in old leaves.

Fig. 3. PAGE of total leaf nucleic acids from sunblotch infected symptomless carrier trees after treatment with ribonuclease A in 0.1 SSC buffer for 10 min at 25 °C. Concentrations of ribonuclease were: (a) 0, control; (b) 0.01 μg/ml; (c) 0.1 μg/ml; (d) 1.0 μg/ml; (e) 10.0 μg/ml. Note that SB-RNA was digested at 1 μg/ml (compare with Fig. 2) but single stranded nucleic acids, including the 12S component, were digested at 0.1 μg/ml (c).
Sensitivity of SB-RNA to nucleases

Total nucleic acids from leaves of symptomless carrier trees were treated with ribonuclease A in SSC buffer at 25 °C for 10 min at various concentrations, and the samples were then electrophoresed in 2.5% gels. In untreated samples, plant ribosomal RNA, 4S and 5S and SB-RNA were all present (Fig. 2a). Ribonuclease A at a concentration of 0.01 μg/ml had no effect (Fig. 2b) but at 0.1 μg/ml, the ssRNAs were digested (Fig. 2c). The SB-RNA was not digested at 1.0 μg/ml (Fig. 2d) but at 10 μg/ml most of the SB-RNA had been degraded (Fig. 2e). In 0.1 SSC buffer (Fig. 3), ssRNAs were again degraded at a ribonuclease concentration of 0.1 μg/ml (Fig. 3c) whilst SB-RNA remained intact. However, in this low salt buffer, SB-RNA was digested by 1 μg/ml ribonuclease (Fig. 3d); this is in contrast to its partial resistance to the enzyme in high salt buffer even at concentrations of 10 μg/ml of ribonuclease. This indicates that SB-RNA is more resistant to ribonuclease A than single-stranded nucleic acids and that this resistance is salt dependent, the SB-RNA being more resistant to ribonuclease A in high salt than in low salt buffers. The stability of 4S and 5S RNA in the presence of ribonuclease A is a reflection of their secondary and tertiary structure and their small molecular size (Cramer, 1971; Crouch, 1976).

When total leaf nucleic acids were treated at 25 °C for 1 h with deoxyribonuclease at 10 μg/ml in SSC buffer containing 1 mM-MgCl₂, plant DNA was degraded but SB-RNA remained unaffected.

Effect of heating on SB-RNA

Nucleic acid samples in SSC buffer were heated at 70 °C for 10 min or at 100 °C for 3 min and either chilled rapidly in ice or cooled slowly at room temperature. The electrophoretic mobility of the SB-RNA was not affected by any of these treatments although in some experiments an additional band was detected at about 6.5S when nucleic acids were heated at 100 °C and chilled rapidly. When nucleic acid samples, previously heated to 100 °C and chilled in ice, were treated with ribonuclease A (1 μg/ml at 25 °C for 10 min in SSC buffer) the SB-RNA band was not present, indicating that denaturation of the nucleic acid by heating had reduced its resistance to the ribonuclease.

Solubility in lithium chloride

Ethanol-precipitated total leaf nucleic acids were resuspended in distilled water and LiCl was added to a final concentration of 2 M. The solution was left overnight at 4 °C and then centrifuged at 10000 g for 10 min to remove insoluble nucleic acid species. Nucleic acids soluble in LiCl were precipitated with ethanol, collected by centrifugation, dried under vacuum and dissolved in RS buffer. After PAGE, the single-stranded nucleic acids (ribosomal RNA) were not detected but the SB-RNA band was still present showing that, unlike the ssRNAs, SB-RNA is soluble in 2 M-LiCl.

Mol. wt. determinations

Initial experiments to determine the mol. wt. of SB-RNA were carried out under non-denaturing conditions. The mobility of the nucleic acid in gels was determined using all E. coli ribonucleic acids as markers in the 2.5% to 5% gels. The mol. wt. of SB-RNA calculated from a plot of log mol. wt. versus mobility (Fig. 4a) varied from 56000 to 71000 (Table 1). In 20% gels, SB-RNA migrated as a sharp band with an apparent mol. wt. of 50000. In gel concentrations of 2.5% to 5%, the average value for 15 determinations was 65000 with a standard deviation of ±5000. CSV, electrophoresed in the same slab gel at concentrations of 2.5, 4 and 5%, migrated more slowly than SB-RNA and appeared to have a mol. wt. of around 90000 to 110000.
Table 1. Determination of sunblotch RNA apparent mol. wt. in polyacrylamide gels of various concentrations under non-denaturing and denaturing conditions

<table>
<thead>
<tr>
<th>Polyacrylamide (%)</th>
<th>Conditions</th>
<th>Mol. wt.</th>
<th>Mol. wt. of CSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>Non-denaturing</td>
<td>63000</td>
<td>90000</td>
</tr>
<tr>
<td>4</td>
<td>Non-denaturing</td>
<td>71000</td>
<td>110000</td>
</tr>
<tr>
<td>5</td>
<td>Non-denaturing</td>
<td>66000</td>
<td>90000</td>
</tr>
<tr>
<td>20</td>
<td>Non-denaturing</td>
<td>50000</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>Formalin, denaturing*</td>
<td>115000</td>
<td></td>
</tr>
<tr>
<td>8 M-urea, denaturing†</td>
<td></td>
<td>112000</td>
<td></td>
</tr>
</tbody>
</table>

* Boedtker, 1971.

The mol. wt. of SB-RNA was also obtained in formalin gels under denaturing conditions to minimize the effects of conformation on mobility (Boedtker, 1971). SB-RNA migrated as a single sharp band with a mobility equivalent to a mol. wt. of 115000 (Fig. 4b; average of two determinations). In 8 M-urea gels at 60 °C, SB-RNA migrated as a single sharp band with a mobility equivalent to a mol. wt. of 112000 (average of two determinations).
Table 2. Location of sunblotch RNA in subcellular fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Concentration of SB-RNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1:</td>
<td></td>
</tr>
<tr>
<td>10000 g pellet</td>
<td>+ + +</td>
</tr>
<tr>
<td>100000 g pellet</td>
<td>-</td>
</tr>
<tr>
<td>Supernatant</td>
<td>+</td>
</tr>
<tr>
<td>Experiment 2:</td>
<td></td>
</tr>
<tr>
<td>'Nuclear'</td>
<td>+</td>
</tr>
<tr>
<td>'Chloroplast'</td>
<td>+ + +</td>
</tr>
<tr>
<td>'Mitochondrial'</td>
<td>-</td>
</tr>
<tr>
<td>'Cytoplasmic'</td>
<td>-</td>
</tr>
</tbody>
</table>

* Symbols represent: + + +, intensely stained SB-RNA band on gels, intensity equivalent to that for 5S RNA; +, trace amounts of SB-RNA; -, no SB-RNA band detected.

**Location of SB-RNA in subcellular fractions**

In an initial experiment, the distribution of SB-RNA between the 10000 g pellet, 100000 g pellet and supernatant was studied. Most of the SB-RNA was in the low speed pellet, none was detected in the high speed pellet and only a trace was left in the supernatant (Table 2). This indicated that the SB-RNA was associated either with membranes or with the larger cell organelles. After a more detailed fractionation of the cell contents, most of the SB-RNA was found in the 'chloroplast' fraction (Table 2) with a small amount in the 'nuclear' fraction. No SB-RNA was found in either the 'cytoplasmic' or 'mitochondrial' fractions. The 'chloroplast' fraction also contained material released from membranous cellular fragments by Triton X-100 and therefore the SB-RNA was associated with either the chloroplast or endoplasmic reticulum.

**DISCUSSION**

The causal agent of sunblotch disease has not yet been identified. No micro-organisms have been found to be associated with it, nor have any virus-like particles been observed in thin sections of infected tissues or in extracts from such tissues. The only possible pathogen that has been associated with sunblotch disease has been SB-RNA (Dale & Allen, 1979; Thomas & Mohamed, 1979) although pathogenicity has not been proved.

The occurrence of SB-RNA in bark lesions of infected symptom-bearing trees and its absence from the leaves of these trees suggests that the expression of symptoms is a hypersensitive reaction which confines the causal agent within the lesion. In symptomless carrier trees, however, there is no hypersensitive reaction and consequently the SB-RNA is not localized and can be readily detected in the leaves. This would also explain why the rate of seed transmission is so high in symptomless carrier trees (86 to 100 %) and low in symptom-bearing trees (less than 5 %). The cause of the hypersensitive reaction and its occurrence in some trees but not in others remains unknown at present.

The present study adds weight to the view that SB-RNA has many properties in common with the known viroids (Diener et al. 1977). The electrophoretic mobility of SB-RNA under non-denaturing conditions is similar to that of the known viroids; SB-RNA migrating slightly faster than CSV in polyacrylamide gels. The mol. wt. of SB-RNA determined under non-denaturing conditions is similar to that of cadang-cadang RNA (Randles et al. 1976) but appears to be greater than that of potato spindle tuber viroid (PSTV; Diener, 1974). Although SB-RNA migrated as a sharp band in 20 % gels, the mol. wt. determination cannot be considered reliable as only two markers, 4S and 5S RNA, were available and under the conditions employed (electrophoresis for 5 h) the nucleic acids had only migrated.
a short distance into the gel. The mol. wt. determined under denaturing conditions, in both formalin and urea gels, was 112,000 to 115,000. This value is considerably higher than that obtained in non-denaturing conditions and differs from the values for cadang-cadang (Randles et al. 1976) and PSTV (Diener, 1974) also obtained under denaturing conditions. However, this value is comparable to that calculated for the mol. wt. of PSTV (approx. 120,000) from the nucleotide sequence (Gross et al. 1978).

The resistance of SB-RNA to ribonuclease A at high salt concentrations and its partial resistance at lower salt concentrations indicates that the SB-RNA is either a double-stranded molecule or else a single-stranded molecule with extensive base-pairing. It appears that SB-RNA is not a double stranded molecule since heating to 100 °C and chilling rapidly in ice did not affect its mobility in gels; under these conditions a double stranded molecule would yield two separate strands of lower mol. wt. than the native RNA (Pinder et al. 1974). These results are consistent with SB-RNA having a structure similar to PSTV, that is, a covalently closed circle (Gross et al. 1978).

Initial experiments indicated that SB-RNA was associated with cellular material that sedimented in the 10,000 g pellet as is the case with other viroids (Diener, 1974). In further tissue-fractionation experiments, SB-RNA appeared to be associated specifically with the ‘chloroplast’ fraction, i.e. the material released from chloroplasts and other membrane-bound fragments by treatment with Triton X-100; this fraction would have included material from the endoplasmic reticulum. Very little SB-RNA was found in the ‘nuclear’ fraction; this is in marked contrast to PSTV which is mainly associated with the chromatin in the nuclear fraction (Diener, 1971). On the other hand, citrus exocortis viroid (CEV) has been shown to be associated not only with the nucleus, but also with the endomembrane system (Semancik et al. 1976). Therefore it appears that SB-RNA, like CEV but unlike PSTV, is associated both with nuclear material and with the endoplasmic reticulum of plant cells.

The final proof that SB-RNA is a viroid would be to demonstrate that the isolated nucleic acid is infectious and causes symptoms of the disease when transmitted to healthy plants. There are two main obstacles to this: the disease agent has not yet been mechanically transmissible and the time taken for symptoms to appear in graft indexing vary from 4 months to 4 years. Experiments to transmit the disease using purified nucleic acid have been initiated recently but until results from these are available, Koch’s postulates cannot be demonstrated. A similar situation occurred with cadang-cadang of coconuts where the presence of a viroid-like RNA was reported (Randles, 1975) some time before infectivity was demonstrated (Randles et al. 1977). However, the similarity of the properties of SB-RNA to those of the known viroids would justify the hypothesis that sunblotch of avocados is a viroid disease.

These findings are of considerable importance at the practical level. Until now, symptomless carriers of sunblotch disease could be detected only by a laborious graft indexing procedure requiring considerable effort and a minimum period of 2 years. Detection of sunblotch RNA in extracts from leaves of symptomless carrier trees is much simpler, and requires a period of only 4 days. This should greatly facilitate disease surveys, as well as other types of research on sunblotch in avocado.
Avocado sunblotch viroid-like RNA

REFERENCES


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