Multiplication of Cucumber Pale Fruit Viroid in Inoculated Tomato Leaf Protoplasts

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(Accepted 31 December 1976)

SUMMARY

Protoplasts were isolated from leaves of three tomato cultivars and inoculated with citrus exocortis viroid (CEV), potato spindle tuber viroid (PSTV) and cucumber pale fruit viroid (CPFV), respectively, using glycine-KOH-buffered mannitol at pH 9. CPFV multiplied detectably in protoplasts from the tomato cultivar Hilda 72 but replication of the other two viroids was rarely detectable. The minimum viroid concentration for infection was 10 μg RNA/ml using 10⁶ protoplasts/ml. Newly synthesized viroid RNA was detected 36 h after inoculation by bioassay, and 48 h after inoculation by ³H-uridine incorporation into the viroid RNA band obtained by polyacrylamide gel electrophoresis. Incorporation of radioactive uridine into CPFV in inoculated protoplasts reached about 0.6% of that into tRNA during 72 h after inoculation. In protoplasts isolated from systemically infected leaves, however, incorporation of ³H-uridine into CPFV was only 0.1% of that into tRNA. In inoculated protoplasts, incorporation of ³H-uridine into CPFV was much more rapid at 35°C than at 25°C but incorporation into tRNA was similar at the two temperatures.

INTRODUCTION

Recent investigations with highly purified preparations have revealed that viroids are uncoated, single-stranded, covalently closed circular RNA molecules, with molecular weights of 107000 to 127000 which exist in their native state as highly base-paired rod-like structures (Sänger et al. 1976; Henco, Riesner & Sänger, 1977; Sänger & Riesner, 1977). These RNAs are known to be pathogenic to certain higher plants, causing the following diseases: potato spindle tuber (Diener, 1971); citrus exocortis (Sänger, 1972; Semancik & Weathers, 1972); cucumber pale fruit (Van Dorst & Peters, 1974); chrysanthemum stunt (Hollings & Stone, 1973; Diener & Lawson, 1973), and chrysanthemum chlorotic mottle (Romaine & Horst, 1975). There is indirect evidence that the cadang-cadang disease of coconut is also caused by a viroid (Randles, 1975; Randles, Rillo & Diener, 1976).

Viroids are replicated in the infected host cell although their potential genetic information is not sufficient to code for a protein with a molecular weight larger than 10000. Thus, unlike conventional RNA viruses, viroids cannot code directly for a viroid-specific RNA polymerase. Consequently, the mechanism of viroid replication is not yet understood. Recent attempts to elucidate the mode of viroid replication in leaf strips (Diener & Smith, 1975), and in isolated nuclei (Takahashi & Diener, 1975) from systematically infected tomato have shown that potato spindle tuber viroid replication is inhibited by actinomycin D.
From these results the involvement of host DNA during viroid replication was inferred. However, these systems have severe experimental limitations, the most important being their unsuitability for the study of primary infection, and the sequence of ensuing cellular processes.

Isolated protoplasts from tobacco have been successfully used to investigate the primary events after inoculation with particles of tobacco mosaic virus (TMV) (Sakai & Takebe, 1974; Aoki & Takebe, 1975; Paterson & Knight, 1975) or with isolated plant virus RNA (Aoki & Takebe, 1969; Motoyoshi et al. 1973). We recently demonstrated replication of citrus exocortis viroid in protoplasts isolated from systemically infected tomato leaves (Mühlbach, Camacho-Henriquez & Sänger, 1977). Tomato protoplasts may therefore also be a promising system to study the cellular processes after infection with viroid RNA. In this report we describe the conditions for the infection of tomato protoplasts with viroid RNA and for successful viroid replication. A short account of this work has been presented previously (Sänger & Mühlbach, 1976).

METHODS

Preparation of protoplasts. The tomato plants (Lycopersicon esculentum cv. Hilda 72, Rutgers, and Rentita) were grown in a greenhouse, and prepared for protoplast isolation as described elsewhere (Mühlbach et al. 1977). Protoplasts were isolated aseptically from tomato leaves according to the method of Motoyoshi & Oshima (1975) with the following modifications: leaves were cut into 1 mm strips instead of their lower epidermis being removed; 0.78 M-mannitol was used as osmoticum instead of 0.7 M-mannitol; 0.5 \% potassium dextran sulphate proved to be more satisfactory than 0.1 \%.

Inoculation of protoplasts with viroid RNA. Three different viroid species were used in this study: those causing potato spindle tuber disease (PSTV), citrus exocortis disease (CEV), and cucumber pale fruit disease (CPFV). All viroids were propagated in cv. Rentita tomato plants, isolated and purified by a large-scale procedure as previously described (Sänger & Ramm, 1975). The isolated tomato protoplasts were inoculated with viroid RNA according to the 'pH 9 procedure' described for the inoculation of tobacco protoplasts with TMV-RNA by Sarkar, Upadhya & Melchers (1974). The inoculation medium consisted of: 0.1 M-glucose; 0.78 M-mannitol; 0.1 M-sucrose; 0.1 M-NaCl; 5 mM-MgCl₂; and 0.2 µg/ml poly-L-ornithine (mol. wt. about 150000, Pilot Chemical Inc.), with the pH adjusted to 9.0 with 1 N-KOH.

Viroid RNA, dissolved in twice-distilled water, was added to 10 ml of the autoclaved inoculation medium to give a final concentration of 10 µg/ml. After sterilization by Millipore filters (0.45 µm), the inoculum was shaken for 20 min at 30 °C in a waterbath. Freshly prepared protoplasts (10⁷) were pelleted, resuspended in the viroid-containing inoculation medium, and incubated in a waterbath for 30 min at 30 °C. As controls, tomato protoplasts were treated in the same way with the inoculation medium, but without viroid RNA. After incubation, protoplasts were sedimented by centrifugation, washed four times with 0.78 M-mannitol containing 10⁻⁴ M-CaCl₂ and incubated at a density of 5 × 10⁴ protoplasts/ml in the culture medium of Motoyoshi & Oshima (1975), supplemented with 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l thiamin-HCl, 1 mg/l naphthalene acetic acid, 1 mg/l 6-benzyladenine and 0.78 M-mannitol, the pH being adjusted to 7.0 with 1 N-KOH. Cephaloridine (300 µg/ml) and nystatin (25 units/ml) were added to prevent microbial growth. Continuous light of about 1500 lux was given by two Osram L-Fluora tubes throughout the period of culture. Unless otherwise stated, the ambient temperature, which was con-
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trolled by a waterbath, was usually 28 °C. For incorporation studies, 0.5 μCi/ml 2-14C-
uridine (specific activity 55 mCi/mmoll, CFA 315, Radiochemical Centre, Amersham) was
added to uninfected protoplasts, whereas 10 μCi/ml 5-3H-uridine (specific activity 25 to 30
Ci/mmoll, RTK 178, Radiochemical Centre) was added to viroid-inoculated protoplasts
immediately after they had been resuspended in the culture medium. Samples (10 ml) were
harvested after 24, 48, 72 and 96 h of culture by centrifuging at 100g for 2 min. The proto-
plast pellets were washed twice with fresh culture medium and stored at −80 °C after
shock-freezing in liquid nitrogen.

Extraction of RNA and polyacrylamide gel electrophoresis. Nucleic acids were extracted
from frozen protoplasts with the aid of phenol, and fractionated with 2 M-LiCl as described
for nucleic acids from intact tomato leaves (Sänger & Ramm, 1975). Polyacrylamide gel
electrophoresis of the RNA soluble in 2 M-LiCl, and determination of radioactivity was
performed exactly as described by Mühlbach et al. (1977).

Estimation of viroid biosynthesis. The synthesis of viroid RNA was estimated from
3H-incorporation into the viroid band in the gels. Since the concentration of viroid RNA
in our experiments was too low to be determined spectrophotometrically, specific radio-
activity could not be calculated. Therefore, the true rate of viroid biosynthesis could not be
determined, and we express viroid biosynthesis as incorporation into viroid RNA as a
percentage of incorporation into tRNA.

Synthesis of viroid RNA was also determined by bioassay. For the zero time control
a portion of protoplasts was sedimented by centrifuging at 2 h after inoculation, washed
twice with fresh culture medium to remove remaining traces of the inoculum, shock-frozen
and stored at −80 °C until RNA extraction. Total nucleic acids were extracted from
unlabelled protoplasts with phenol-SDS at 2, 24, 36 and 48 h after inoculation, and pre-
cipitated with ethanol in the cold. The nucleic acids from samples of 5 × 106 to 1 × 107
protoplasts were dissolved in 2 ml of 0.1 M-phosphate buffer, pH 7.5, and used as inoculum.
Each sample was bioassayed on 24 cv. Rutgers tomato plants (7 to 10 days old) by in-
oculating the leaves with a few drops of inoculum, using carborundum and a glass spatula.
The number of infected plants was first recorded after three weeks when most of the infected
plants had developed the typical epinasty, leaf distortion and stunting. All plants that had
not developed symptoms at this time were decapitated and the final reading of the bioassay
was made after 8 weeks. Under these conditions the newly developing axillary shoots of any
infected plants showed obvious symptoms even at very low concentrations of viroid in the
inoculum. Infectivity is expressed as the number of symptom-bearing plants over the total
number of inoculated plants. The data given are mean values from three experiments.

Inoculation of tomato plants with CPFV. For isolation of protoplasts from systemically
CPFV-infected tomato leaves, the leaves of 7-day-old cv. Hilda 72 tomato plants were
inoculated with a buffer homogenate from symptom-bearing leaves of cv. Rentita systemi-
cally infected with CPFV. Protoplasts were isolated as described above from cv. Hilda 72
leaves that had developed during the three weeks after inoculation. Because plants of this
cultivar do not develop symptoms despite intensive viroid synthesis, leaf homogenates were
bioassayed on cv. Rutgers tomato in order to assure the presence of viroid in the leaves
used as a source of protoplasts.
Table I. Influence of tomato cultivars and viroid species on viroid biosynthesis in whole plants and in inoculated protoplasts

<table>
<thead>
<tr>
<th>Tomato cultivar</th>
<th>Average number of viable protoplasts obtained per g leaf material</th>
<th>Symptoms in plants 4 weeks after inoculation</th>
<th>µg viroid RNA per kg infected leaf material</th>
<th>3H-uridine incorporation index</th>
<th>Viroid synthesis in 10^7 protoplasts*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rentita</td>
<td>0.3 x 10^6</td>
<td>CEV: + +</td>
<td>80</td>
<td>NT†</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PSTV: + +</td>
<td>125</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPFV: + +</td>
<td>180</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Rutgers</td>
<td>1 x 10^6</td>
<td>CEV: + ++</td>
<td>70</td>
<td>--</td>
<td>0/24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PSTV: + ++</td>
<td>110</td>
<td>--</td>
<td>4/24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPFV: + ++</td>
<td>200</td>
<td>--</td>
<td>0/24</td>
</tr>
<tr>
<td>Hilda 72</td>
<td>5 x 10^6</td>
<td>CEV: --</td>
<td>100</td>
<td>±</td>
<td>3/24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PSTV: --</td>
<td>140</td>
<td>+</td>
<td>9/24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPFV: --</td>
<td>220</td>
<td>++</td>
<td>14/24</td>
</tr>
</tbody>
</table>

* Viroid biosynthesis was determined by bioassay 48 h after inoculation and by 3H-uridine incorporation into viroid RNA 72 h after inoculation.
† NT, not tested.

RESULTS

Influence of tomato cultivars and viroid species on viroid synthesis in isolated protoplasts

The tomato cultivar Hilda 72 proved to be more suitable for the isolation of protoplasts from leaf tissue than the cultivars Rentita or Rutgers (Table 1, first column). We consistently obtained about five to ten times more viable protoplasts from cv. Hilda 72 leaves than from Rutgers leaves. The sturdy bush tomato cv. Rentita, which is routinely used in our laboratory as a convenient host for mass propagation of viroids, proved to be unsuitable as a source of protoplasts and was therefore not tested further. Although cv. Hilda 72 plants do not show symptoms of viroid infection after mechanical inoculation with the three viroid species tested, viroid biosynthesis is readily demonstrated when leaf material from inoculated plants is subjected to our routine procedure used for the isolation and purification of viroid RNA (Sänger et al. 1976). The yield of viroid RNA was determined from excised viroid bands obtained after two successive separations on preparative polyacrylamide gels. It appears (Table 1, third column) that with 100 to 200 µg viroid RNA per kg leaf material viroid biosynthesis is more active in cv. Hilda 72 leaves than in those of the cultivars used for bioassay (Rutgers), or for mass propagation (Rentita) of viroids, provided that during culture the plants are held at greenhouse temperatures of 30 to 35°C.

Viroid biosynthesis after in vitro inoculation was studied in protoplasts from leaves of cv. Rutgers and Hilda 72. In pilot experiments the minimum viroid concentration for CPFV infection was found to be about 10 µg viroid RNA per 10^6 protoplasts suspended in 1 ml inoculation medium. A period of 30 min incubation was optimal, and any longer exposure to the alkaline inoculation medium caused increasing deterioration and shrinkage of protoplasts. Similar effects were produced when the alkaline inoculation medium contained 2.5% KCl plus 1% MgCl₂·6H₂O instead of mannitol, as used by Sarkar et al. (1974) for in vitro inoculation of tobacco protoplasts by TMV-RNA. It is interesting to note that under these conditions of inoculation a considerable amount of viroid infectivity was detected 24 and 36 h after inoculation. However, after prolonged culture, infectivity decreased rapidly and the protoplasts disintegrated as could be seen by microscopy. The
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Fig. 1. Analysis in a 5% polyacrylamide gel containing 8 M-urea of the 2 M-LiCl-soluble RNA from 14C-uridine labelled uninfected tomato protoplasts and from 3H-uridine labelled protoplasts inoculated in vitro with CPFV-RNA at 24, 48 and 72 h after inoculation. Unlabelled purified CPFV-RNA (10 μg) was coelectrophoresed with the 72 h sample and the gel scanned at 260 nm, and stained with methylene blue to locate the viroid band precisely. The inset represents the 'viroid region' of the gels on an expanded scale. ○—○, 3H radioactivity; •—•, 14C radioactivity; ——, E\textsubscript{260}. 
Table 2. Incorporation of \(^3\)H-uridine and \(^{14}\)C-uridine into CPFV and transfer RNA in tomato (cv. Hilda 72) protoplasts

<table>
<thead>
<tr>
<th>Period of labelling (h)</th>
<th>Viroid region (^{(3})H d/min)</th>
<th>tRNA region (^{(3})H d/min)</th>
<th>Relative incorporation* (^{(3})H d/min)</th>
<th>Viroid region (^{(14})C d/min)</th>
<th>tRNA region (^{(14})C d/min)</th>
<th>Relative incorporation (^{(14})C d/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1152</td>
<td>0.173</td>
<td>865.0</td>
<td>0.057</td>
<td>2.980</td>
<td>0.204</td>
</tr>
<tr>
<td>48</td>
<td>17,043</td>
<td>0.141</td>
<td>121,726</td>
<td>0.109</td>
<td>89.39</td>
<td>0.156</td>
</tr>
<tr>
<td>72</td>
<td>40,918</td>
<td>0.107</td>
<td>1,007,723</td>
<td>0.647</td>
<td>60,501</td>
<td>0.035</td>
</tr>
</tbody>
</table>

* Relative incorporation = \(\frac{\text{d/min viroid RNA} \times 100}{\text{d/min tRNA}}\).

inoculation medium containing 0.78 M-mannitol as osmotic stabilizer did not show this detrimental effect, and was therefore used in our standard procedure.

When cv. Hilda 72 protoplasts were inoculated with the three viroid species, only CPFV replicated reproducibly as assessed by bioassay and by \(^3\)H-uridine incorporation. No \(^3\)H-peak of viroid RNA could be detected in RNA from protoplasts inoculated with PSTV or CEV after polyacrylamide gel electrophoresis, but in some tests a small amount of viroid infectivity was detected. Protoplasts from cv. Rutgers could be infected by PSTV, but after inoculation with CEV and CPFV no newly synthesized viroid RNA was detectable by bioassay (Table 1, last column).

Time course of viroid replication

In order to study the appearance of newly synthesized viroid RNA in protoplasts infected in vitro, the combination of CPFV and Hilda 72 protoplasts was used. The fractions of RNA soluble in 2 M-LiCl from \(^{14}\)C-labelled uninfected, and \(^3\)H-labelled viroid-infected protoplasts were pooled, and separated on 5% polyacrylamide gels in the presence of 8 M-urea at 12 to 15 °C gel temperature. Under these conditions viroid RNA is clearly detectable as a distinct peak between the cellular 5S RNA and 7S RNA species. As shown in Fig. 1 the peak of newly synthesized \(^3\)H-labelled viroid RNA appeared 48 h after inoculation. As expected, the more sensitive bioassay for viroid infectivity revealed newly synthesized viroid RNA 36 h after inoculation but not earlier. This lag was observed despite the fact that biosynthesis of cellular RNA occurs in our system immediately after isolation and inoculation of protoplasts, as shown by the incorporation of labelled uridine into ribosomal RNA, 7S RNA, 5S RNA and tRNA within 24 h after inoculation. The failure in detecting newly synthesized viroid RNA at this time may be due to a rather low rate of viroid biosynthesis.

Viroid biosynthesis, calculated as the percentage of incorporation of \(^3\)H-uridine into viroid RNA compared to tRNA, increased from zero to about 0.65% 72 h after inoculation of protoplasts with CPFV (Table 2). This means more than a 1000-fold increase of incorporation into viroid RNA between 24 and 72 h after inoculation compared to the approximately 100-fold increase of incorporation into tRNA. In contrast, in protoplasts isolated from Hilda 72 leaves systemically infected with CPFV, the relative amount of incorporation into viroid RNA compared to tRNA was only 0.1% after 72 h of culture in the presence of radioactive uridine (Table 2). In no case was a \(^{14}\)C peak observed at the position of the \(^3\)H-labelled viroid RNA, when RNA from uninfected \(^{14}\)C-labelled protoplasts was coelectrophoresed with RNA from \(^3\)H-labelled viroid-infected protoplasts.
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Temperature dependance of viroid biosynthesis

In previous experiments we showed that the biosynthesis of PSTV in tomato plants, and of CEV in *Gynura aurantiaca* is greatly enhanced by raising the temperature to around 35 °C, whereas the biosynthesis of cellular nucleic acids is little affected (Sänger & Ramm, 1975). We therefore studied the effect of suboptimal (25 °C), and optimal (35 °C) temperatures on viroid biosynthesis in protoplasts inoculated *in vitro* with CPFV. In these experiments the samples of inoculated protoplasts were divided in two portions which were then cultured in the presence of $^3$H-uridine for 72 h at 25 °C and 35 °C, respectively. Three major effects were found: (1) incorporation of $^3$H-uridine into viroid RNA for 48 h was about 10 times greater at 35 °C than at 25 °C (Fig. 2a); (2) between 48 and 72 h, incorporation into viroid RNA increased exponentially at 25 °C but slowed greatly at 35 °C, and microscopy showed that protoplasts at 35 °C gradually deteriorated during this period (Fig. 2a); (3) the amount of incorporation into cellular 5S RNA and tRNA likewise stopped after 48 h at 35 °C, whereas at 25 °C it increased until 72 h (Fig. 2b). A plausible explanation of these findings is that CPFV synthesis in inoculated protoplasts is more rapid at 35 °C than at 25 °C.

**DISCUSSION**

The results described show that protoplasts from tomato leaves can be infected by inoculation *in vitro* with CPFV, and that the ensuing viroid replication can be followed by measuring the incorporation of $^3$H-uridine into viroid RNA. The tomato protoplasts became infected using an inoculation medium of alkaline pH that was previously found
to be optimal for in vitro infection of tobacco protoplasts with minimal concentrations of TMV-RNA (Sarkar et al. 1974). In experiments using tomato protoplasts and TMV-RNA (unpublished results) we also found this ‘pH 9’ inoculation medium to be superior to the citrate-buffer medium of pH 5.2 used by Motoyoshi et al. (1973).

Tomato protoplasts seem to be much more sensitive to osmotic conditions than tobacco protoplasts. The saline osmoticum (2.5 % KCl plus 1 % MgCl₂·6H₂O) as used by Sarkar et al. (1974) for inoculation of tobacco protoplasts with TMV-RNA could not be used for tomato protoplasts, which were rapidly damaged in this medium. The best osmoticum appeared to be 0.78 M-mannitol, which was found to maintain the stability of tomato protoplasts during isolation, culture and inoculation.

Studying protoplasts from two tomato cultivars and three different viroid species we found only one combination, namely Hilda 72 protoplasts and CPFV, to be suitable for detailed investigation of viroid replication. There is only one explanation we can offer for this finding at present: Hilda 72 protoplasts may withstand the procedures of isolation and inoculation better than protoplasts from Rutgers or Rentita tomato leaves. The pronounced difference between the three viroid species with respect to their ability to infect protoplasts and to initiate viroid replication is not yet understood. It is unlikely to be caused by differences in specific infectivity of the viroids tested, because for each we found that 50 to 100 RNA molecules are sufficient to infect one tomato plant (Sänger et al. 1976). Because CPFV reaches the highest yields in intact tomato plants, we assume that this viroid is the best adapted for replication in tomato. Accordingly, we tend to explain our results with different viroids in Hilda 72 protoplasts in rather general terms of differences in host adaptation.

Viroids do not initiate the biosynthesis of a coat protein nor has any other viroid-specific protein been found in infected tissue. Therefore, the percentage of infected protoplasts cannot be determined on the basis of fluorescent antibody staining, in contrast to conventional plant viruses (Otsuki & Takebe, 1969). Moreover, we did not observe any cytopathic effect in the light microscope that could be used for this purpose. It is not even possible to guess the expected percentage of infected protoplasts by indirect comparison. Sarkar et al. (1974) reported that with their pH 9 inoculation procedure they were able to infect more than 90 % of their tobacco protoplasts with TMV-RNA, whereas Suzuki & Takebe (1976) obtained only 3.5 %, using exactly the same system and the same technique. The efficiency of different inoculation procedures could therefore only be determined indirectly by bioassaying the viroid RNA synthesized by the whole protoplast population. Despite these special technical limitations the system studied is well suited to investigation of viroid replication with radioactive precursors.

Although we could follow viroid biosynthesis by incorporation of labelled uridine into viroid RNA after in vitro inoculation of protoplasts, we are not yet able to calculate the rate of viroid replication. As has been pointed out recently (Sutton & Kemp, 1976), the absolute rates of RNA biosynthesis can only be determined by analysis of the uptake of an appropriate radioactive compound into a precursor pool and by the incorporation of the precursor into the product. Therefore, it is imperative that the specific radioactivity of the nucleoside triphosphate precursors of RNA and of the RNA itself be measured as a function of time. In the case of viroid replication in protoplasts, the total amounts of the precursor and of the viroid RNA synthesized are too low to be determined spectrophotometrically. Thus, neither the specific radioactivity of viroid RNA nor the specific radioactivity of the nucleoside triphosphates incorporated into viroid RNA can be calculated at present. Therefore, we determined the increase of viroid RNA in relative terms using ³H-uridine
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incorporation into newly synthesized viroid RNA as a percentage of the $^3$H-uridine incorporation into newly synthesized tRNA. Probably the biosynthesis of tRNA takes place in most, if not all, of our protoplasts, whereas viroid replication can only occur in the still unknown fraction of infected protoplasts. Accordingly, our approach can only provide an underestimate of the actual viroid biosynthesis occurring in the individual infected cell. However, we consider this to be a reasonable measure of viroid replication. Both nucleic acids are well separated in the same gel, and the biosynthesis of tRNA is a reliable parameter of a well functioning cellular metabolism. As judged from these determinations, viroid biosynthesis seems to be more active in protoplasts inoculated in vitro than in protoplasts isolated from systemically infected leaves. Moreover, in protoplasts inoculated in vitro the percentage of labelled viroid RNA increased to 0.65% of labelled tRNA within 72 h after inoculation, whereas in protoplasts from systemically infected leaves the percentage of labelled viroid RNA never exceeded the initial value of about 0.1% of labelled tRNA during 72 h. This finding supports our previous conclusion that viroid concentration may have reached a plateau in cells of systemically infected leaves, and synthesis not resumed after isolation of protoplasts (Mühlbach et al. 1977). These results indicate that only protoplasts inoculated with viroid RNA in vitro are suitable for the investigation of the mechanism of viroid replication at the cellular level.

Our previous finding that viroid replication in intact tomato plants is inordinately temperature dependent (Sänger & Ramm, 1975) is compatible with the results now obtained using tomato protoplasts. Interestingly, viroid biosynthesis continued at 35 °C even beyond 48 h after inoculation in protoplasts which did not synthesize tRNA and 5S RNA after this time of culture at the ‘high temperature’. The correlation between higher temperatures and increasing viroid biosynthesis may reflect the adaptation of viroid isolates to their natural growth conditions, because all viroid diseases known at present are confined to plants growing in subtropical, tropical and continental climates, or in greenhouses.

The protoplast-viroid system described in this report opens new perspectives for the investigation of the replication mechanism of viroids at the cellular level. The use of suspension cultures of single tomato cells, lacking their cellulose wall, and amenable to infection with viroid RNA may help to overcome the limitations inherent in the use of whole plants, intact leaves and leaf discs.

We wish to thank Mrs K. Ramm, Mrs M. Bahr and Mrs I. Schlag for capable technical assistance. This work was supported by the Sonderforschungsbereich 47 (Virologie).

REFERENCES


(Received 15 October 1976)