Infection of Tobacco Mesophyll Protoplasts by Potato Virus X

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SUMMARY

Conditions favouring the infection of isolated tobacco mesophyll protoplasts by potato virus X (PVX) were studied in detail, and a procedure to effect infection in 70% of protoplasts was developed. PVX required higher concentrations of both inoculum virus and poly-L-ornithine than tobacco mosaic virus (TMV) or cucumber mosaic virus (CMV). PVX infection showed a unique response to varying pH of the inoculation medium. The time course of PVX multiplication in protoplasts resembled that of TMV. Unlike the infection by TMV and CMV, PVX infection was partially inhibited by actinomycin D. Inclusion bodies characteristic for PVX infection were present in the infected protoplasts, but their involvement in virus production appeared to be unlikely.

INTRODUCTION

Studies on tobacco mesophyll protoplasts inoculated in vitro with tobacco mosaic virus (TMV) (Takebe & Otsuki, 1969) revealed that the great majority of cells in the system are infected simultaneously (Otsuki et al. 1972a). This unique feature of the protoplast system helped greatly in the succeeding work to provide better insights into the processes of TMV infection and multiplication (Otsuki, Shimomura & Takebe, 1972b; Sakai & Takebe, 1972). Tobacco mesophyll protoplasts were subsequently inoculated with plant viruses of spherical morphology (Otsuki & Takebe, 1971, 1973a; Motoyoshi et al. 1973), and the same feature was exploited to study ultrastructural aspects of cucumber mosaic virus (CMV) infection (Honda et al. 1973). An attempt to develop a similar system for viruses of filamentous morphology led to the inoculation of tobacco mesophyll protoplasts with potato virus X (PVX) (Otsuki & Takebe, 1972). We now report in detail the conditions which favour the infection of protoplasts by this virus. The course of PVX multiplication in protoplasts was also studied by infectivity assay, by an immunological method and by electron microscopy.

METHODS

Virus and virus purification. PVX-no. 5, a ringspot strain of potato virus X (Oshima, 1959), was obtained from Dr N. Oshima of this Institute and was used throughout this study. After the fifth single-lesion transfer on Gomphrena globosa L., the virus was inoculated on to Nicotiana tabacum L. cv. Xanthi nc, and systemically infected leaves with typical symptoms were used as the source of inoculum for further virus propagation. Purification of PVX was carried out according to the method of Shepard & Secor (1969) with several modifications.
Inoculated leaves of Xanthi nc were harvested 12 days after inoculation and stored at \(-70°C\) until use. After being thawed, about 100 g of infected leaves were homogenized in a blender with 100 ml of cold distilled water containing 0.1% thioglycolic acid (pH adjusted to 7.0). The homogenate was filtered through sheets of gauze and n-butanol was added to 10% (v/v). After standing with occasional stirring for 10 h, the homogenate was centrifuged at 10000 g for 15 min to remove denatured host materials. NaCl and polyethylene glycol (No. 6000, Wako Pure Chemical Industries, Ltd) were then dissolved in the supernatant fraction to 2% (w/v) and 7% (w/v), respectively. The mixture was allowed to stand for 1 h and then centrifuged at 8000 g for 20 min. The supernatant fluid was discarded and the precipitate was dissolved in 20 ml of distilled water. The virus solution was dialysed overnight against water, and was then centrifuged at 12000 g for 20 min. The supernatant fluid was further purified by two cycles of differential centrifuging at 105000 g for 60 min and at 12000 g for 20 min. All the operations were carried out at 4°C and the purified virus dissolved in water was stored at the same temperature. The virus preparation had an $E_{260}/E_{247}$ ratio of 1.15 to 1.20 and an $E_{260}/E_{280}$ ratio of 1.16 to 1.18. The concentration of virus was calculated using the extinction index reported by Reichmann (1959). The procedure described above yielded roughly four times more purified PVX than the original procedure of Shepard & Secor (1969), the final yield ranging from 200 to 350 mg per kg of infected tissues.

Plants. Tobacco plants (Nicotiana tabacum L. cv Xanthi) were grown in a greenhouse in which the temperature was maintained between 20 °C and 28 °C throughout the year. Light from white fluorescent lamps was supplied to maintain a 15 h day length in winter. Plants were grown in 15 cm pots with a 4:1 mixture of soil and leaf compost and were weekly given an artificial fertilizer (3:1:1 mixture of ammonium sulphate, calcium superphosphate and potassium chloride). Leaves freshly expanded to full length (20 to 25 cm) of 55 to 60- (in summer) or 70 to 80- (in winter) day-old plants were used as the source of protoplasts.

Isolation of protoplasts. Protoplasts of palisade parenchyma cells were isolated essentially according to the method previously described (Takebe, Otsuki & Aoki, 1968; Aoki & Takebe, 1969). The lower epidermis of detached leaves, which had partially lost turgor, was peeled off with forceps, and the stripped leaves were cut into pieces of approx. 9 cm². The leaf pieces were first macerated with polygalacturonase to dissociate the mesophyll tissue into free cells. The maceration medium contained 0.5% (w/v) Macerozyme ME-45 (Kinki Yakult Manufac. Co., Nishinomiya, Japan) and 0.5% (w/v) potassium dextran sulphate (sulphur content 17.8%, mol. wt. of source dextran about 560, Meito Sangyo Co., Nagoya, Japan) in 0.7 M-mannitol, pH being adjusted to 5.8 with HCl and KOH. About 2 g (fresh weight) of stripped leaf pieces were soaked in 20 ml of the maceration medium in 100 ml Erlenmeyer flask, and were infiltrated for 1.5 min using a rotary vacuum pump. The flask was then shaken reciprocally (stroke 4.5 cm) in a water bath of 25 °C at a frequency of 120 excursions/min. After 3 to 5 min of shaking, the maceration medium containing broken cells and debris from the cut edges of leaf pieces was discarded and was replaced by 20 ml of fresh maceration medium. Shaking for further 7 to 10 min released spongy parenchyma cells into medium, which was again discarded and replaced by fresh medium. Final shaking for 25 to 30 min dissociated the mesophyll tissue nearly completely, releasing palisade parenchyma cells into medium. The medium was passed successively through a plastic net and a sheet of gauze to remove upper epidermis and veins which remained undigested.

Palisade parenchyma cells thus obtained were collected by centrifuging for 1 min at 200 g and were suspended in 20 ml of 2% (w/v) Cellulase Onozuka R-10 (Kinki Yakult Manufac. Co.) in 0.7 M-mannitol at pH 5.2. The suspension was incubated for 30 to 60 min at 36 °C with reciprocal shaking at a frequency of 80 excursions/min. The resulting proto-
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Plast suspension was divided into 10 ml portions, and protoplasts were collected and washed 3 times with 0.7 M-mannitol solution by centrifuging for 1 to 2 min at 130 g. Dead cells and their debris were largely eliminated during washing.

Inoculation of protoplasts. For the inoculation of protoplasts with PVX, the procedure previously developed for inoculating TMV (Takebe & Otsuki, 1969; Otsuki et al. 1972a) was modified in detail to assure maximal infection by PVX. Purified PVX was dissolved at a concentration of 10 µg/ml in 10 ml of 0.02 M-potassium citrate buffer, pH 5.8, containing D-mannitol to 0.7 M and poly-l-ornithine (mol. wt. 130000, Pilot Chemicals, Inc., Watertown, Massachusetts, U.S.A.) to 6 µg/ml. After the solution was kept for 20 min at 25 °C, it was mixed with 10 ml of freshly resuspended protoplasts (approx. 1×10⁶ cells/ml in 0.7 M-mannitol solution), and the mixture was kept for 10 min at 25 °C. The final concentration of virus and poly-l-ornithine was thus 5 µg/ml and 3 µg/ml, respectively. The protoplasts were then separated from unadsorbed virus by low speed centrifuging, washed and incubated at 28 °C as described previously for TMV-inoculated protoplasts (Otsuki et al. 1972b).

Staining with fluorescent antibody. Fluorescein isothiocyanate-labelled antibody to PVX was prepared using the method previously reported for TMV (Otsuki & Takebe, 1969). The fluorescent antibody thus obtained had a titre of 1/2024 as measured by precipitin ring test, and a dye/protein molar ratio of 2.75 as estimated from the E₂₄₀ and E₂₈₀. PVX-infected protoplasts were stained using 1/16 or 1/32 dilution of the conjugated antibody following the method for staining TMV-infected protoplasts (Otsuki & Takebe, 1969), except that 95 % ethanol was used for fixation (Otsuki et al. 1972b). The fluorescent PVX antibody did not stain protoplasts infected by TMV or CMV. PVX-infected protoplasts were not stainable with fluorescent antibody to TMV or CMV.

Infectivity assay. PVX infectivity in protoplasts was assayed using Gomphrena globosa L. leaves. Protoplasts were collected by centrifuging at 150 g for 2 min, washed once with 0.7 M-mannitol solution and stored at −70 °C. After being thawed, the protoplasts were suspended in 5 ml of 0.1 M-phosphate buffer, pH 7.0, containing 0.1 % (v/v) thioglycolic acid. The suspension was homogenized for 1 min in a VirTis Micro Homogenizer at the top speed. Cell debris were removed by centrifuging at 2000 g for 5 min, and the supernatant fluid was inoculated without dilution on the leaves of 50 to 60 day-old G. globosa L. using cotton swabs. The plants had been trimmed leaving the 5th and 6th pairs (from bottom) of opposite leaves. The lesions produced were counted after 4 days.

Electron microscopy. Protoplasts infected by PVX were processed for electron microscopy according to the method already reported (Takebe et al. 1973).

RESULTS

Effects of inoculation conditions on frequency of PVX infection

The procedure adequate for the inoculation of tobacco mesophyll protoplasts with TMV (Otsuki et al. 1972a) gave rise to PVX infection in only a small fraction of protoplasts (less than 10 %). Conditions in the individual steps of the inoculation procedure were, therefore, examined in detail for their effects on the frequency of PVX infection. In the experiments of this type, the frequency of infection was determined by scoring the number of protoplasts which were stainable with fluorescent PVX antibody 28 to 44 h after inoculation.
Effects of pH of the inoculation medium on the frequency of PVX infection are illustrated in Fig. 1. Larger numbers of protoplasts were infected as pH was raised from 5.0 to 5.8. At higher pH’s the frequency of infection tended to decrease slightly.

Inoculum concentration

Frequency of infection was proportional to the logarithm of PVX concentration within a range of 0.5 to 5 μg/ml (Fig. 2). No improvement in the frequency of infection was obtained by increasing the virus concentration above 5 μg/ml.

Concentration of poly-L-ornithine

As has been reported for other viruses (Takebe & Otsuki, 1969; Motoyoshi et al. 1973; Otsuki & Takebe, 1973a) infection of protoplasts by PVX was also highly dependent on the presence of poly-L-ornithine. Practically no infection occurred when this polycation was omitted, and the number of infected protoplasts increased with increasing concentrations of poly-L-ornithine up to 3 μg/ml (Fig. 3). Inoculum virus concentration in this experiment was also 3 μg/ml. With this virus concentration, poly-L-ornithine above 3 μg/ml caused some damage to protoplasts and decreased infection frequency.

Pre-incubation of virus with poly-L-ornithine

Infection frequency was improved by pre-incubating virus and poly-L-ornithine prior to their addition to protoplasts (Fig. 4). In other experiments of the same type, very few protoplasts were infected when virus and poly-L-ornithine were added to protoplasts without pre-incubation. Pre-incubation for 20 min was sufficient for the maximal level of infection (Fig. 4).
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Fig. 3. Effect of poly-L-ornithine concentration on the frequency of PVX infection. Protoplasts were inoculated with 3 µg/ml PVX in the presence of various poly-L-ornithine concentrations. Conditions were otherwise as described in Methods, except virus/poly-L-ornithine pre-incubation was for 10 min.

Fig. 4. Effect of PVX/poly-L-ornithine pre-incubation on the frequency of PVX infection. Protoplasts were inoculated with PVX as described in Methods except that the length of virus/poly-L-ornithine pre-incubation was varied as indicated.

Duration of contact of virus with protoplasts

Fig. 5 shows the results of an experiment in which protoplasts were incubated for varying periods with PVX/poly-L-ornithine mixture which had been pre-incubated for 20 min. It can be seen that 5 min contact of virus and protoplasts is enough to give maximal infection.

These results were compiled to construct an optimal inoculation procedure which is described in Methods. Fifty to 70% of protoplasts were infected using this procedure depending on lots of protoplasts and inoculum virus.

PVX multiplication in protoplasts

Time course

The course of PVX multiplication in tobacco mesophyll protoplasts was followed by assaying the infectivity in protoplasts at intervals after inoculation. The results illustrated in Fig. 6 show that the virus multiplied very rapidly in the first 24 h, during which the infectivity in protoplasts increased 1000-fold. The rate of virus multiplication slowed down markedly in later periods. Although the overall time course of PVX multiplication in the protoplasts was similar to that of TMV (Otsuki et al. 1972b), an initial drop of infectivity, which possibly reflects uncoating of the infecting particles (Takebe & Otsuki, 1969), could not be clearly demonstrated. This is probably due to limited sensitivity and accuracy of PVX assay.

Accumulation of virus antigen

PVX antigen was first detectable in the protoplasts 12 h after inoculation by fluorescent antibody staining. At this stage weak fluorescence was found in some areas of the cytoplasm. At 24 h or later, fluorescence was much more intense and was distributed throughout the
Fig. 5. Effect of time of contact between virus and protoplasts on the frequency of PVX infection. Conditions for inoculation were as described in Methods except that the time of incubation of protoplasts with virus was varied as indicated.

Fig. 6. Time course of PVX multiplication in protoplasts. Protoplasts inoculated with PVX were suspended in 100 ml of incubation medium at a cell density of $5 \times 10^5$ ml and were incubated in 10 ml portions. One flask each was harvested at indicated times, extract of protoplasts was prepared and assayed for infectivity. Each point represents the mean of twelve determinations. Staining of 48 h sample with fluorescent antibody showed that 39.2% of protoplasts were infected. Crosses show data from a separate experiment in which 47.1% of protoplasts were infected and the cell density during incubation was $2.6 \times 10^5$ ml.

Fig. 7. Fluorescence micrograph of protoplasts stained with fluorescent PVX-antibody 24 h after inoculation. The protoplast in the middle contains specific fluorescence.

cytoplasm (Fig. 7). Unlike TMV or CMV antigen in protoplasts, PVX antigen showed always more or less diffuse staining.

Effects of inhibitors

Two types each of the inhibitors of RNA and protein synthesis were selected to see their effects on PVX multiplication in protoplasts. These inhibitors were added to the medium in which the inoculated protoplasts were cultured, and their effects were studied by assaying
PVX infection of tobacco protoplasts

Table 1. Influence of inhibitors of RNA and protein synthesis on PVX multiplication in protoplasts*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (μg/ml)</th>
<th>0 h</th>
<th>24 h</th>
<th>48 h</th>
<th>Percentage infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>1.1 ± 1.7</td>
<td>342 ± 172</td>
<td>504 ± 138</td>
<td>47.1</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-Thiouracil</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>200</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Protoplasts inoculated with PVX were suspended in 80 ml of incubation medium at a density of 2.6 × 10⁵/ml. Ten ml was removed as 0 h sample and the rest was incubated in 10 ml portions for 24 and 48 h with and without the addition of inhibitors.

† Number of lesions produced by the extract of protoplasts on a leaf of Gomphrena globosa L. Mean of seven determinations with standard deviation.

‡ After 48 h of incubation, protoplasts were stained with fluorescent antibody. Percentage of the protoplasts containing virus antigen was determined under a fluorescence microscope.

Table 2. Effects of actinomycin D added at various times after inoculation*

<table>
<thead>
<tr>
<th>Hours elapsed between start of culture and addition of actinomycin D</th>
<th>Percentage infection†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>65.1</td>
</tr>
<tr>
<td>0</td>
<td>19.1</td>
</tr>
<tr>
<td>3</td>
<td>56.9</td>
</tr>
<tr>
<td>6</td>
<td>63.0</td>
</tr>
<tr>
<td>9</td>
<td>66.1</td>
</tr>
<tr>
<td>12</td>
<td>66.0</td>
</tr>
</tbody>
</table>

* Inoculated protoplasts were cultured at a density of 2.5 × 10⁵/ml in 10 ml portions, and actinomycin D was added to the medium at 10 μg/ml at various times after the start of culture.

† Percentage of protoplasts stainable with fluorescent PVX antibody 32 h after the start of culture.

the infectivity produced as well as by determining the number of infected protoplasts (Table 1). Under these conditions, actinomycin D markedly suppressed PVX multiplication during the first 24 h, although nearly as much infectivity was produced later in the presence of this inhibitor as in its absence (Table 1). Actinomycin D also significantly reduced the number of protoplasts accumulating PVX antigen (Table 1). However, the amount of PVX antigen in the individual infected protoplasts, as judged from the amount of fluorescence, appeared to be larger 48 h after inoculation in the presence than in the absence of actinomycin D. Effect of actinomycin D was also studied by adding the drug at different times after inoculation (Table 2). It can be seen that actinomycin D failed to reduce the number of virus producing protoplasts when added 6 h after inoculation or later.

2-Thiouracil did not markedly reduce the number of protoplasts stainable with fluorescent PVX antibody (Table 1). However, the fluorescence in stained protoplasts was much less intense than in the protoplasts cultured without inhibitor. The results of infectivity assay also show that 2-thiouracil inhibits virus multiplication. A high concentration of chloramphenicol had no influence on PVX multiplication in protoplasts, while cycloheximide completely suppressed virus production (Table 1). These results clearly show that the cytoplasmic ribosome system and not the chloroplast ribosome system is responsible for the synthesis of PVX proteins.
Fig. 8. PVX particles produced in tobacco mesophyll protoplasts 24 h after inoculation. ER, endoplasmic reticulum; P, plasmalemma; T, tonoplast; V, virus.

Fig. 9. Aggregate of PVX particles formed in tobacco mesophyll protoplasts 72 h after inoculation. P, plasmalemma; V, virus.

Fig. 10. Inclusion bodies formed in PVX-infected protoplasts. Protoplasts were fixed 24 h after inoculation. I, inclusion body; V, virus.
Electron microscopy

PVX multiplication in protoplasts was also followed by examining thin sections at various times after inoculation. A number of PVX particles scattered in the cytoplasm appeared 14 h after inoculation. The number of virus particles increased rapidly during the subsequent hours, but at 24 h they were still dispersed in the matrix of cytoplasm (Fig. 8). At 48 h or later, the virus particles congregated into large aggregates excluding cellular components (Fig. 9). The formation of large virus aggregates apparently caused enlargement of cytoplasmic volume, of which major portion was occupied by the virus aggregates. PVX particles were not found in the nucleus, chloroplasts or other organelles which remained structurally normal at least until 96 h after inoculation.

Shalla & Shepard (1972) reported a detailed study on the inclusion bodies characteristic for PVX infection. The same structures as described by these workers were found in the protoplasts containing large numbers of PVX particles. When the inoculated protoplasts were cultured for 96 h, the inclusion bodies disintegrated in the later periods of infection. Virus particles were sometimes associated with the inclusion bodies in such a way that might suggest the involvement of these structures in virus production (Fig. 10). This possibility seems to be unlikely, however, because the inclusion bodies usually appeared after a large amount of virus was already formed. Furthermore, there were also infection experiments in which active virus multiplication was not accompanied by the occurrence of such inclusion bodies.

DISCUSSION

Isolated tobacco mesophyll protoplasts were successfully inoculated with PVX, a filamentous virus, by a brief contact of virus and protoplasts in the presence of poly-L-ornithine. Although the procedure used was basically the same as that originally devised for inoculating TMV (Takebe & Otsuki, 1969) or CMV (Otsuki & Takebe, 1973a), some details in the procedure were modified for maximal infection by PVX. For example, the pH optimum for the infection by PVX was found to be considerably higher than pH's at which TMV and CMV are inoculated. The response of PVX infection toward varying pH (Fig. 1) was entirely different from that of TMV or CMV; the frequency of infection by the latter viruses was highest at pH's near 5.0 and decreased as the pH of inoculation medium was raised (Y. Otsuki & I. Takebe, unpublished results, Otsuki & Takebe, 1973a). Some efforts were made to correlate the behaviour of the three viruses at different pH's with the electric charge of virus particle. TMV and CMV used in our studies had an isoelectric point of 3.83 and 5.90, respectively (Y. Otsuki, unpublished data). Assuming that the protoplast surface has a net negative charge (Grout et al. 1972), the response of infection by these viruses to pH may be explained by the decrease in negative or the increase in positive charge of virus particle at lower pH's, which should facilitate virus adsorption to protoplasts. Unfortunately, the isoelectric point of PVX could not be determined by titration, because for unknown reasons, this virus failed to precipitate at any pH tested. A reasonable explanation of the response of PVX infection to pH awaits further investigation.

Inoculum virus concentration required for maximal infection by PVX was 5 μg/ml (Fig. 2). A same level of infection by TMV, whose particle weight is similar to that of PVX, could be attained at a virus concentration of 0.1 μg/ml (Otsuki & Takebe, 1973a). The difference in the efficiency of infection between PVX and TMV may be explained by the inherent difference in specific infectivity of these viruses. It should be noted that the higher virus concentration necessitated a higher poly-L-ornithine concentration; 1 μg/ml of poly-L-ornithine,
which was sufficient for the infection by TMV and CMV, was suboptimal for PVX infection (Fig. 3). This finding suggests that a part of poly-L-ornithine is used for complexing virus particles. The fact that the pre-incubation of virus with poly-L-ornithine stimulates infection (Fig. 4) not only supports this interpretation, but also indicates that the formation of virus/poly-L-ornithine complex is a necessary step for the infection of protoplasts. The rate at which such an interaction proceeds differs apparently according to the virus, since PVX infection required a longer virus/poly-L-ornithine pre-incubation than TMV infection. It is clear from the results in this and previous studies that different viruses require different inoculation conditions for infecting protoplasts at high frequencies. The failure of Coutts, Cocking & Kassanis (1972) to inoculate tobacco mesophyll protoplasts with PVX may be due either to improper inoculation conditions or to lower specific infectivity of virus, or to both.

Previous studies with electron microscopy showed that TMV (Hibi & Yora, 1972; Otsuki et al. 1972a) and CMV (Honda et al. 1973) enter the protoplasts by a pinocytic process. It was desirable to see whether a similar process is involved in the penetration of PVX into protoplasts, particularly because a different mechanism was recently proposed for the virus uptake by protoplasts (Burgess, Motoyoshi & Fleming, 1973). However, the small width and the flexible nature of this particular virus made it extremely difficult to identify infecting particles in thin sections, and so far it has not been possible to obtain pictures which clearly show PVX particles entering the protoplasts.

PVX multiplication as studied by infectivity assay (Fig. 6) followed a time course similar to that of TMV. Although no quantitative estimation was made, a high yield of virus per cell is suggested by the electron microscopic observations (Fig. 9). An interesting aspect of PVX infection of protoplasts was that, unlike the infection by other viruses studied (Takebe & Otsuki, 1969; Otsuki & Takebe, 1973a), it was sensitive to actinomycin D. The results obtained in this study (Tables 1 and 2) indicate that actinomycin D interferes with the process leading to the onset of PVX multiplication but not with the virus multiplication per se once it was initiated. It may, therefore, be suggested that an early step in PVX infection depends on the product of a host gene.

Conditions for the synchronous infection of protoplasts at satisfactory levels were established now for plant viruses of three major morphological groups. The protoplast system is not only useful for the studies of individual viruses but also provides an opportunity to investigate at a cellular level mixed infection by two or more different viruses (Otsuki & Takebe, 1973b).

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REFERENCES


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