Isolation and Characterization of Subviral Structures from Tomato Spotted Wilt Virus

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SUMMARY

Particles of a stable isolate of tomato spotted wilt virus (TSWV) had a density of 1.147 g/ml in sucrose and contained four major structural proteins of mol. wt.: 1, 26 \times 10^3; 2, 52 \times 10^3; 3, 56 \times 10^3; and 4, 78 \times 10^3. The RNA which was not infectious consisted of four segments of mol. wt.: 1, 3.6 \times 10^6; 2, 2.8 \times 10^6; 3, 2.0 \times 10^6; and 4, 1.3 \times 10^6. The ribonucleoprotein (RNP) of TSWV, prepared by treatment of purified virus with Nonidet P-40, could be separated into three components by sedimentation on sucrose density-gradients. All three components contained protein 1; the slowest sedimenting component also contained RNA species 1, 2 and 4, the second component contained RNA species 1 and 2 and the fastest sedimenting component contained all four RNA species. The three components were slightly infectious and infectivity was enhanced by pooling these components. The RNP, when observed under the electron microscope, appeared to consist of strands.

Enveloped particles devoid of the outer layer of projections were obtained by treating TSWV with bromelain. These subviral particles retained about 1% of their original infectivity, had a density of 1.137 g/ml in sucrose and only contained protein 1. It was concluded that this protein forms the subunit of the nucleocapsid and that TSWV, unlike the majority of enveloped viruses, does not possess an internal membrane protein.

INTRODUCTION

Tomato spotted wilt virus (TSWV) is a structurally complex virus composed of nucleoprotein bounded by a lipid envelope, the surface of which is covered by projections or spikes (Ie, 1970). TSWV has been classified into a monotypic group on the basis of its nucleic acid and protein composition (Fenner, 1976) although it bears some structural resemblance to the myxoviruses. Mohamed et al. (1973) found that TSWV contains three major structural proteins (mol. wt. 84 \times 10^3, 50 \times 10^3 and 27 \times 10^3), whilst Tas et al. (1977) reported four major structural proteins (mol. wt. 78 \times 10^3, 58 \times 10^3, 52 \times 10^3 and 29 \times 10^3). The smallest structural protein (mol. wt. 27 \times 10^3) is associated with the nucleic acid (Mohamed et al., 1973), while iodination experiments suggested that the other structural proteins are located outside the lipid envelope (Tas et al., 1977). An infectious ribonucleoprotein (RNP) component was isolated from TSWV by treatment with Nonidet P-40 (NP-40) (Mohamed et al., 1973; Van den Hurk et al., 1977), but no detailed studies were carried out on the composition of this component. Van den Hurk et al. (1977) found that the genome of TSWV consists of four RNA segments of mol. wt. 2.7 \times 10^6, 1.9 \times 10^6, 1.7 \times 10^6 and 1.3 \times 10^6.

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An isolate of TSWV from New Zealand was found to be relatively stable and easy to
purify so that sufficient quantities of purified virus were available for characterization studies.
This study was therefore initiated to isolate and characterize: (i) particles devoid of the outer
layer of spikes obtained by treatment of virus with proteolytic enzymes and (ii) the RNP of
TSWV.

METHODS

Virus and plants. An isolate of TSWV from Solanum aviculare Forst. f., a native New
Zealand species, was propagated in Datura stramonium L. plants grown in an insect-free
glasshouse maintained at 20 to 25 °C. Virus infectivity was assayed on Nicotiana tabacum L.
cv. White Burley.

Purification of virus. Purified virus was prepared as described by Mohamed et al. (1973)
except that a second sucrose density-gradient centrifugation step was included.

Enzyme treatments. Virus preparations were treated with chymotrypsin (1 mg/ml) or
bromelain (2-5 mg/ml) in 1 mm-sodium phosphate buffer, pH 7, containing 0.1% (v/v)
mercaptoethanol. After incubation under the required conditions, the treated samples were
layered on to 10 to 40% sucrose gradients prepared in 1 mM-phosphate buffer, pH 7,
containing 0.01 M-NaESO3; gradients were centrifuged for 45 to 90 min at 24000 rev/min in
a Spinco SW25 rotor and then fractionated using an ISCO density-gradient fractionator and
u.v. analyser. All studies (electron microscopy, infectivity and analyses of protein
composition) on enzyme-treated virus were carried out after purification on sucrose
density-gradients.

Analysis of virus proteins. Purified virus particles were dissociated into polypeptides by
adding an equal volume of 0.1 m-sodium phosphate buffer, pH 7.2, containing 10 m-urea,
2% SDS and 0.1% mercaptoethanol and heating at 100 °C for 3 min. Protein samples were
analysed by electrophoresis on 7.5% polyacrylamide gels (Mohamed, 1978).

Electron microscopy. Samples on carbon-collodion-coated copper grids were negatively
stained with unbuffered 2% uranyl acetate and examined in a Jeol 100B electron microscope
at 80 kV.

Preparation of RNP. RNP was prepared from purified TSWV by disrupting virus particles
by the addition of NP-40 to a final concentration of 0.5%. After incubation at 0 °C for 2
min, the suspension was layered on to 10 to 40% sucrose density-gradients containing 1
mm-sodium phosphate, 0.01 M-Na2SO3, pH 7, and centrifuged for 6 h at 24000 rev/min in a
Beckman SW25 rotor.

Infectivity of RNP. Fractions corresponding to the RNP components separated on sucrose
gradients were collected and centrifuged for 10 h at 40000 rev/min in a Spinco 40 rotor.
Each fraction was resuspended in 1 ml sterile buffer (1 mm-sodium phosphate, 0.01
M-Na2SO3, pH 7); 0.5 ml of each fraction was used for inoculation and the other 0.5 ml was
pooled.

The infectivity of unfractionated RNP was determined by treating purified virus with
NP-40 and centrifuging for 2 h at 50000 rev/min to sediment the RNP. The pellet was
resuspended in sterile buffer and inoculated on to tobacco leaves, using untreated virus at the
same dilution as controls.

Preparation of virus nucleic acid. For infectivity assays and gel electrophoresis studies,
viral nucleic acid was extracted from purified TSWV by the phenol–SDS method (Van den
Hurk et al., 1977). The final nucleic acid pellet was resuspended in sterile resuspension (RS)
buffer (0.04 m-tris-acetate, 0.02 m-sodium acetate, 2 mm-EDTA, 0.2% SDS, 5% sucrose,
pH 7.5) for electrophoresis, or in SSC buffer (0.15 M-NaCl, 0.015 M-sodium citrate) for
infectivity assays. Nucleic acid was prepared from the RNP components either by the
single-phase phenol–SDS method (Diener & Schneider, 1968) or by disrupting the RNP in
Fig. 1. U.v. absorption traces of 10 to 40% sucrose density-gradients containing (a) untreated TSWV; (b to d) TSWV treated with bromelain at: (b) 20 °C for 15 min, (c) 37 °C for 15 min and (d) 37 °C for 30 min. Gradients were centrifuged for 90 min at 24000 rev/min. TSWV treated with bromelain at 0 °C for 15 min sedimented at the same rate as untreated virus shown in (a).

Fig. 2. Electron micrograph of purified TSWV after one cycle of sucrose density-gradient centrifugation. Arrows indicate particles with an outer layer of projections clearly visible.

1% SDS, 0.5% mercaptoethanol, 0.05 M-sodium phosphate, 1 mM-EDTA, 10% sucrose, pH 7.4 (Brakke & Rochow, 1974) at 60 °C for 5 min. Nucleic acids were fractionated on 2% acrylamide–0.5% agarose slab gels using the tris–phosphate–SDS buffer system (Loening, 1968); electrophoresis and staining were carried out as described by Mohamed (1978).

RESULTS

Properties of purified TSWV

After two cycles of sucrose density-gradient centrifugation, partially purified preparations of TSWV formed a single well-defined light-scattering band (Fig. 1a). These preparations were highly infectious but were pale green in colour, indicating the presence of host plant contaminants. The density of purified virus in sucrose was determined by centrifugation in quasi-equilibrium sucrose density-gradients (Sarkar et al., 1976). Purified preparations of TSWV were layered on to 10 to 60% linear sucrose gradients in 1 mM-sodium phosphate, 0.01 mM-Na₂SO₃, pH 7, and centrifuged at 24000 rev/min for 4 h at 4 °C. The virus formed a single band at a density of 1.147 g/ml; the contaminating plant material formed a diffuse band ranging in density from 1.15 to 1.17 g/ml.
Fig. 3. Analyses of the polypeptides of TSWV on polyacrylamide gels: (a) untreated control; (b to e) bromelain treated at: (b) 0 °C for 15 min, (c) 20 °C for 15 min, (d) 37 °C for 15 min and (e) 37 °C for 30 min.

Purified preparations of TSWV, examined in an electron microscope, were found to contain spherical virus particles 75 to 85 nm in diam. (Fig. 2); there were few or no membranous plant contaminants present as have been reported with other preparations (Mohamed et al., 1973). The virus particles had a well-defined, unstained envelope surrounded by an outer layer of projections (Fig. 2); no internal structure could be differentiated even in particles penetrated by stain.

Analysis of the protein composition of purified TSWV by gel electrophoresis showed that the virus contained four major structural proteins (Fig. 3a). The mol. wt. estimates of these were: 1, 26 × 10³; 2, 52 × 10³; 3, 56 × 10³; and 4, 78 × 10³. These estimates were in agreement with the values reported by Tas et al. (1977) who had determined the mol. wt. using a wide range of gel concentrations. Another protein (labelled 2' in Fig. 3a) was present in most preparations and varied in amount from <1% to 10% of the total virus protein. This protein was also present in preparations from healthy plants, indicating that it may be a host contaminant. The relative proportions of structural proteins 1 to 4 did not vary from one preparation to another. Minor protein components with a high mol. wt. were also detected in most preparations; they formed less than 1% of the total virus protein and it was not possible to get a reliable estimate of their mol. wt. These may be minor structural proteins or aggregates of the major proteins.

Total virus nucleic acid was extracted from TSWV by the phenol–SDS method and inoculated on to tobacco plants in three separate experiments at concentrations of 10, 15 and 20 μg/ml. No infectivity was detected in any of these experiments although analysis of the preparations showed that all the nucleic acid components were present. This confirms results obtained by Van den Hurk et al. (1977) that the RNA of TSWV is not infectious.

Four species of RNA were detected when the nucleic acid of TSWV was extracted by the phenol–SDS method and fractionated on polyacrylamide gels. Their mol. wt., estimated using *Escherichia coli* ribosomal RNA (mol. wt. 1.07 × 10⁶ and 0.56 × 10⁶) and tobacco mosaic virus RNA (2.05 × 10⁶) as markers, were: 1, 3.6 × 10⁶; 2, 2.8 × 10⁶; 3, 2.0 × 10⁶; and 4,
Fig. 4. Electron micrographs of purified TSWV treated with bromelain at: (a) 0 °C for 15 min, (b) 37 °C for 15 min and (c to e) 37 °C for 30 min. Arrows in (b) indicate particles with the lipid envelope clearly visible, also shown in (e).

1.3 x 10^6. These values can only be considered as approximate since no reliable high mol. wt. RNA species were available for use as markers. RNA 1 was the major component and formed about 40% of the total nucleic acid; RNAs 2, 3 and 4 each represented about 20% of the total RNA. These proportions were estimated by a visual assessment of the intensity of the stained bands on gels and are therefore only a rough guide.

Preparation and properties of spikeless subviral particles

Attempts were made to prepare spikeless particles from TSWV by using techniques successful in removing surface projections from other enveloped viruses (Rifkin & Compans, 1971; Schultze, 1972; Sarkar et al., 1976). Treatment of TSWV with HCl or with Pronase resulted in complete loss of infectivity and no virus was recovered. Treatment with chymotrypsin was only partially successful in removing the projections and most of the particles retained all or part of this layer even after prolonged treatment with the enzyme. The method that proved to be successful was treatment with bromelain under certain conditions.

TSWV preparations treated with 2.5 mg/ml bromelain at 0 °C for 15 min sedimented at the same rate as the untreated virus shown in Fig. 1(a). However, the green plant contaminants that normally banded with the virus formed a pellet at the bottom of the tube, while the virus formed a white, light-scattering band. The infectivity of the treated virus was comparable to that of the untreated controls. No differences could be detected between the controls (Fig. 2) and bromelain-treated virus (Fig. 4a) on examination in the electron
microscope. Analysis of the protein composition showed that proteins 1 and 4 were not affected, while proteins 2 and 3 were reduced (Fig. 3b) when compared with the untreated control (Fig. 3a). Two extra proteins, 2a and 2b of mol. wt. $46 \times 10^3$ and $43 \times 10^3$ respectively, were also present and appeared to be degradation products of proteins 2 and 3 formed as a result of bromelain treatment. Protein 2', thought to be a host contaminant, was not detected in any of the bromelain-treated samples (Fig. 3b to e).

After 15 min at 20 °C, bromelain did not affect the sedimentation rate (Fig. 1b) or the structure of the virus, but there was a reduction (about 35%) in infectivity when compared to the untreated controls incubated for 15 min at 20 °C. Gel electrophoresis of the virus proteins (Fig. 3c) showed that protein 2a was not present, protein 2b had increased in quantity relative to the structural proteins and protein 2 was barely detectable, while proteins 3 and 4 were reduced when compared to protein 1.

Bromelain treatment for 15 min at 37 °C reduced the sedimentation rate of TSWV (Fig. 1c) and 90% of the virus infectivity was lost when compared to the control. The layer of projections on the outside of virus particles appeared to be in various stages of degradation: spikeless particles and those with part of the outer layer of spikes missing were observed (Fig. 4b). Gel electrophoresis of virus proteins showed that structural proteins 2 and 3 and protein 2a were not present; protein 2b formed about 10 to 15% of the total virus protein (Fig. 3d).

Treatment with bromelain for 30 min at 37 °C resulted in loss of the projections from all virus particles (Fig. 4c). No light-scattering band was observed on centrifugation in sucrose gradients although a u.v.-absorbing band with a markedly reduced sedimentation rate was detected (Fig. 1d). The infectivity of the spikeless particles was only 1% of that of the control samples. In the electron microscope, only spikeless particles were observed (Fig. 4c, d); these were smooth surfaced and tended to aggregate into clusters (Fig. 4e). In particles penetrated by stain, the lipid envelope was clearly defined (Fig. 4b, c). On sedimentation in quasi-equilibrium sucrose density-gradients, the spikeless particles had a density of 1.137 g/ml, which was slightly lower than that for intact virus and agrees with results obtained with other viruses where loss of the surface projections results in a similar reduction in density (Rifkin & Comamps, 1971). Analysis of the protein composition showed that spikeless particles only contained protein 1 (Fig. 3e), indicating that this protein is protected from digestion by bromelain whereas proteins 2, 3 and 4 are digested by the enzyme.

Analysis of the proteins present in the top fractions of the gradients shown in Fig. 1(b to d) indicated that only one protein, the subunit of bromelain, was present. Presumably, the TSWV spike proteins were digested into small fragments and were not detectable.

There was also a reduction in the amount of virus present in the virus fraction of the gradients after bromelain treatment, especially after treatment at 37 °C for 30 min (Fig. 1d). This loss of virus may be due to disruption of the membrane of some TSWV particles during incubation with the enzyme which would expose any internal protein to digestion by bromelain. The reduction in infectivity (to approx. 1% of the original) and the loss of protein 1 (Fig. 3e) could be due to this loss of particles.

Isolation of RNP

The RNP of TSWV, prepared by disrupting purified virus with NP-40, was separated from the outer layers of the virion and host contaminants by centrifugation through sucrose density-gradients. Under the centrifugation conditions used (6 h at 24000 rev/min) any intact virus would sediment as a pellet. With partially purified virus, four u.v.-absorbing peaks were detected in sucrose gradients (Fig. 5a). In addition, the top one-fifth of the gradient was green in colour and absorbed u.v.; it is presumed that this material consisted of NP-40, plant contaminants and membrane fragments. Virus purified by bromelain treatment (0 °C for 15 min) and treated with NP-40 formed only three u.v.-absorbing bands in sucrose gradients.
Fig. 5. U.v. absorption scans of (a) partially purified and (b) bromelain-treated preparations of TSWV disrupted with NP-40 and centrifuged through sucrose density-gradients. Virus preparations were disrupted by adding NP-40 to 0.5%; the mixture was shaken, stored in ice for 5 min and layered on to 10 to 40% sucrose gradients which were then centrifuged for 6 h at 24000 rev/min in a Beckman SW25 rotor. (Fig. 5 b). Peak 1, present in Fig. 5 (a), was not detected, while the amount of u.v.-absorbing material at the top of the gradient was reduced considerably.

The $A_{260}/A_{280}$ ratios of the four peaks were: peak 1, 1.35; peak 2, 1.39; peak 3, 1.39; and peak 4, 1.23. These results indicate the presence of about 5 to 10% nucleic acid in all four fractions. However, peak 1 was shown not to contain any high mol. wt. nucleic acid, although it may have contained low mol. wt. nucleic acid, which would not be detectable under the electrophoresis conditions employed. Mohamed (1973) showed that preparations of TSWV usually contain 4S and 5S RNA, probably attached as contaminants to the outer layer of the virus particle. The presence of these low mol. wt. RNAs in untreated virus preparations could explain the presence of peak 1 (Fig. 5 a). Bromelain-treated virus, which was relatively free of plant contaminants and hence of the 4S and 5S nucleic acids, had no absorbance at $A_{254}$ in the region of peak 1 (Fig. 5 b).

Fractions corresponding to each of peaks 1 to 4 were collected and inoculated on to eight leaves of *N. tabacum* L. cv. White Burley. The number of lesions formed by these fractions were: peak 1, none; peak 2, 1; peak 3, 4; and peak 4, 7. No infectivity was associated with any other part of the gradient. In the same experiment, the fractions from peaks 2, 3 and 4 were pooled and inoculated on to the test plants: there was a marked increase in infectivity and a total of 59 lesions was formed. Addition of the peak 1 fraction had no effect on the infectivity of the pooled fractions. The low infectivity associated with the individual components may be a result of incomplete separation of the bands under these conditions. In contrast, the infectivity of unfractionated RNP was 20% of that of the untreated control virus.

Fractions corresponding to the four peaks were collected, centrifuged for 10 h at 40000 rev/min in a Beckman 40 rotor, and the pellets resuspended in 1 mM-sodium phosphate plus 10 mM-Na$_2$SO$_4$, pH 7. On examination in the electron microscope, no recognizable structures were observed in the peak 1 fraction. Fractions corresponding to peaks 2, 3 and 4 contained thread-like structures of varying length (Fig. 6 a, b); these may be strands of RNP. Some of these strands appeared to be coiled (Fig. 6 a, b, arrowed) but no definite structure was detectable in the majority of these strands and there was no conclusive evidence of a helical structure. 

Analysis of the protein composition of the material collected from the four peaks showed that fractions corresponding to peaks 2, 3 and 4 contained structural protein 1 only. The peak
Fig. 6. Electron micrographs of the RNP components of TSWV taken from (a) peak 2 and (b) peak 4 after fractionation on sucrose gradients and negative staining with uranyl acetate. Arrows in (a) indicate strands which appear to be coils of material, probably RNP. Arrow in (b) indicates a long, tightly coiled strand.

1 fraction also contained traces of proteins 1 and 4 and the host plant contaminant present in TSWV preparations (protein 2′ in Fig. 3a). This result was confirmed in another experiment where purified virus was treated with 0.5% NP-40, and the RNP was collected by centrifugation at 100,000 g for 2 h. The supernatant was then centrifuged at 100,000 g for 12 h to sediment the envelope fragments. Only protein 1 was detected in the 100,000 g, 2 h pellet (i.e. the RNP fraction) while proteins 2, 3 and 4 and traces of protein 1 were present in the 100,000 g, 12 h pellet (i.e. the membrane fragment fraction). These results indicate that protein 1 is closely associated with the RNP.

RNA was isolated from fractions corresponding to the four peaks and was analysed by polyacrylamide gel electrophoresis. No nucleic acids were detected in peak 1 but peaks 2, 3 and 4 contained the TSWV RNA species in different combinations. Peak 2 contained RNA
species 1, 2 and 4, peak 3 RNA species 1 and 2, and peak 4 all four RNA species. These results suggest that the RNP of TSWV consists of at least three components and that the RNA species are distributed amongst these components.

DISCUSSION

The isolate of TSWV described here is relatively stable and, therefore, it was possible to purify the virus fairly easily. Although the preparations of virus were contaminated with host material, a slight modification of the procedure, i.e. treatment with bromelain at 0 °C for 15 min, resulted in preparations apparently free from these contaminants. Virus prepared in this way did not contain fraction I protein (a major contaminant) or 4S and 5S RNA and retained all or most (more than 95%) of the original infectivity. There was no apparent effect on virus structure, although two structural proteins were partially degraded. Therefore, this method should prove useful for preparing virus suitable for physicochemical studies.

The TSWV isolate described here contains four major structural proteins; this confirms results obtained by Tas et al. (1977). Mohamed et al. (1973) reported only three major proteins in TSWV, although the higher resolution in gels reported here could account for the difference. The discrepancy between the previous reports regarding glycosylation of the structural proteins (Mohamed et al., 1973; Tas et al., 1977) could not be resolved since attempts to stain glycoproteins with Schiff’s reagent resulted in non-specific staining.

The nucleic acid of TSWV was resolved into four species, confirming earlier reports (Van den Hurk et al., 1977) that the RNA is segmented. There are some differences, however, between the RNA composition described here (four species of mol. wt.: $3.6 \times 10^6$, $2.8 \times 10^6$, $2.0 \times 10^6$ and $1.3 \times 10^6$) and that reported by Van den Hurk et al. (1977) who found four species of mol. wt.: $2.7 \times 10^6$, $1.9 \times 10^6$, $1.7 \times 10^6$ and $1.3 \times 10^6$. The $2.0 \times 10^6$ mol. wt. RNA described here probably corresponds to their $1.9 \times 10^6$ and $1.7 \times 10^6$ mol. wt. RNAs since these RNAs could not always be resolved (Van den Hurk et al., 1977). The $3.6 \times 10^6$ mol. wt. RNA reported here may be the RNA 1a which was detected in small quantities by these workers and was also thought to be a protein–nucleic acid complex. However, in this isolate, this RNA is the major constituent of the virus genome. Discrepancies in the relative amounts of the different RNA segments are not surprising, however, as Van den Hurk et al. (1977) had reported considerable variation in the ratios of the four RNA species in virus prepared during different seasons.

The failure to detect any infectivity in RNA preparations from TSWV confirms earlier findings (Mohamed, 1973; Van den Hurk et al., 1977). It also suggests that either the RNA of TSWV does not serve as a mRNA and requires a polymerase for activity or that if it is a mRNA, some factor vital for its activity is missing.

The isolation of an infectious RNP complex agrees with previous results (Mohamed et al., 1973; Van den Hurk et al., 1977), although under the conditions described here it was possible to resolve this complex into three components. All three components contained protein I but differed in their content of the RNA segments; all three were slightly infectious but infectivity was greatly enhanced by pooling the components. These results indicate that the RNP of TSWV may be multi-component in nature as reported with influenza virus (Compans et al., 1972). If a better separation of these components could be achieved, it may be possible to carry out recombination experiments between different strains of TSWV.

The structure of the RNP of TSWV is not clear at present. Earlier reports (Mohamed et al., 1973) had suggested the presence of 'core-like' structures in the RNP complex isolated by treatment with NP-40. In the present study, however, core-like structures were only observed when the RNP complex was not fractionated into components by sucrose density-gradient centrifugation; when the components were separated, the RNP appeared to consist of strands resembling the RNP of influenza virus (Compans et al., 1972). This suggests that, like
influenza virus, the RNP of TSWV may consist of a nucleocapsid with helical symmetry. However, there is no direct evidence to support this suggestion.

Bromelain-treated particles only contained protein 1, indicating that this protein is located within the envelope since it is protected from digestion by the enzyme, unlike proteins 2, 3 and 4 which are presumably located external to the envelope. These results confirm an earlier suggestion by Tas et al. (1977), based on iodination experiments, that protein 1 is located within the envelope. As this protein is also associated with the RNP of TSWV, it can be concluded that it forms the subunit of the nucleocapsid protein. Therefore, unlike the majority of enveloped viruses (Lenard & Compans, 1974), there is no internal membrane protein forming a layer beneath the lipid envelope of TSWV. Interactions between the nucleocapsid protein and the lipid envelope would then be important in stabilizing the virus particles. The presence of ‘core-like’ structures (Mohamed et al., 1973) in preparations of unfraccionated RNP suggests that the RNP of TSWV exists as a compact structure in the intact virion but can be readily separated into discrete strands.

REFERENCES


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