# Biological, antigenic and genomic relationships among the virus isolates causing mosaic disease of sugarcane in South India

**M. Hema[†], M. Venkatramana[†], H. S. Savithri* and P. Sreenivasulu[†,**]**

[†]Department of Virology, Sri Venkateswara University, Tirupati 517 502, India
*Department of Biochemistry, Indian Institute of Science, Bangalore 500 012, India

**The virus isolates causing mosaic disease of commercial sugarcane around Tirupati (Chittoor district, Andhra Pradesh (AP)), Tanuku (West Godavari district, AP), Hospet (Bellary district, Karnataka) and Coimbatore (Tamil Nadu) were propagated on *Sorghum bicolor* cv. Rio by sap inoculation and also through vegetative propagules of sugarcane. In host range studies, the four isolates infected all the 11 tested sorghum differentials with per cent infection ranging from 10 to 100, but they failed to infect *Pennisetum typhoides*, *Zea mays*, *Eleusine coracana* and *Triticum aestivum*. The antigenic relationships among these isolates determined by employing agar gel double diffusion (AGDD), direct antigen coating-enzyme-linked immunosorbent assay (DAC-ELISA) and electroblot immunoassay (EBIA) tests using antiserum of Tirupati (Chittoor district, AP) isolate (sugarcane streak mosaic virus, SCSMV-AP) revealed that the other three isolates are antigenically similar to SCSMV-AP. This was further confirmed by slot-blot hybridization using radioactive nucleic acid probe (pSV-7) specific to 3′-UTR and C-terminal portions of coat protein gene of SCSMV-AP. The heterologous isolates reacted similarly with the probe. The results demonstrated that the virus isolates causing mosaic disease of sugarcane in South Indian states are pathotypes of recently characterized SCSMV-AP, a new member of the proposed genus Tritimovirus of the family Potyviridae.**

SUGARCANE is an important food cum cash crop and is the third largest crop in terms of value next to rice and wheat in India. In India, the crop is grown extensively in Uttar Pradesh, Maharashtra, Punjab, Haryana, Gujarat, Madhya Pradesh, Bihar, Rajastan, Tamil Nadu, Karnataka and Andhra Pradesh[1].

Viruses like sorghum mosaic potyvirus (SrMV), sugarcane badnavirus (SCBV), sugarcane streak geminivirus (SSV), sugarcane Fiji disease fijivirus (FDV) and sugarcane mosaic potyvirus (SCMV) were reported to naturally infect sugarcane in different countries[2,3]. Apart from these viruses, there are reports of a mealybug transmitted clostero-like sugarcane mild mottle virus (SCMMV), an aphid transmitted luteovirus-like agent associated with yellow leaf syndrome (SCYLV) and peanut clump furovirus (sugarcane isolate) (SCRLMV) associated with red leaf mottle of sugarcane from different parts of the world[4–7]. In India, previously unrecorded virus particles ranging from 380 to 460 × 21 nm have been found to be associated with mosaic symptoms in sugarcane[8]. Among these, mosaic disease, a seed piece transmissible disease with interveinal chlorotic specks, streaks or stripes especially on young leaves of sugarcane has been reported to be prevalent in almost all sugarcane cultivating regions of India[9]. Incidence of this virus in commercial fields is nearly 100% in major sugarcane growing states like Madhya Pradesh, Tamil Nadu, Karnataka, Uttar Pradesh, Maharashtra, Andhra Pradesh and Bihar[1]. Even 10–15% yield loss due to this disease is highly significant because of extensive cultivation of the crop.

The family Potyviridae comprising six genera is the largest (30% of all plant viruses) and economically the most important of the plant viruses[10–12]. Members of the family exist in numerous strains or pathotypes which differ in biological properties such as host range or pathogenicity.

Shukla *et al.*[13] were able to classify 17 SCMV and maize dwarf mosaic virus (MDMV) strains from Australia and USA into four distinct potyviruses, namely Johnson-grass mosaic virus, MDMV, SrMV and SCMV. It has been suggested that all these viruses should be grouped under the subgroup sugarcane mosaic virus in the genus Potyvirus[14]. Later, Yang and Mirkov[15] developed group-specific primers for use in RT-PCR-based RFLP analysis for rapid discrimination between strains of SCMV and SrMV. The sequence data that cover the 3′ non-coding region (3′-NCR), coat protein and part of nuclear inclusion b (NIb) genes of four German isolates of sugarcane showed that they were strains of SCMV[16]. Comparison of HPLC peptide profiles of coat protein of two Cuban isolates with Australian and American strains revealed that the Cuban isolates are two different potyviruses[17]. The status of some of the SCMV strains described from the USA (MDMV-C, SCMV-F, SCMV-G, SCMV-K and SCMV-L) and other countries is not known. It is possible that some of these strains may not belong to the four viruses of SCMV subgroup[18,19].

The literature survey indicates that the mosaic disease was caused by different strains of SCMV, which is regarded as one of the most important viruses worldwide in sugarcane growing countries[9,20]. In India, occurrence of different strains of SCMV mainly based on symptoms of natural hosts, reactions on differential hosts and serology were reported[21–35]. None of them has been characterized at the molecular level. Recently, molecular characterization of a virus causing mosaic disease of sugarcane around Tirupati, Chittoor district, Andhra Pradesh, revealed that it is not a strain of SCMV subgroup but is a

**For correspondence.

strain of a new virus named sugarcane streak mosaic (SCSMV) which has been claimed to be a member of Tritimovirus, a proposed genus in the family Potyviridae. The virus was therefore named as SCSMV-AP isolate[36].

Molecular data on the coat protein and genome of Indian virus isolates causing mosaic disease of sugarcane may clarify interviral relationships with other potyviruses infecting cereals and millets. Since the virus isolate (SCSMV-AP) causing mosaic disease of sugarcane in Andhra Pradesh is distinct from the SCMV subgroup, further studies may reveal whether the same or different virus isolates cause mosaic disease in other states in India.

Even though sugarcane is extensively grown as a commercial crop in South India and the mosaic disease is so common, the virus isolates causing disease have not been properly characterized and typed. Hence in the present study, we report the biological, antigenic and molecular relationships among the virus isolates causing mosaic disease of sugarcane in South India.

Sugarcane leaf and stem samples showing mosaic symptoms collected from Tirupati (SCSMV-AP), Tanuku (West Godavari district, AP), Hospet (Bellary district, Karnataka) and Coimbatore (Tamil Nadu) were propagated on *Sorghum bicolor* cv. Rio plants by periodical sap inoculation[30,36]. The four virus isolates were also maintained on sugarcane through vegetative propagules (setts) periodically.

The following test plants were raised from their respective healthy seeds (Prasad, ICRISAT, Patancheru). The seeds were sown in earthen pots containing garden soil and farmyard manure (3 : 1). They are *Sorghum almum* (S.88), *S. caudatum* (IS.12730), *S. cernuum* (IS.1054), *S. nigricans* (IS.8887), *S. verticilliflorum* (S.1), *S. halepense* (S.77), *S. saccharum* (IS.2866), *S. controversum* (S.189), *S. bicolor* cv. Atlas (IS.671), collier (IS.649), Sart (IS.685), *Pennisetum typhoides* (CC.75, PPBS1), *Zea mays* var. local, *Eleusine coracana* (PPR 2709, 2681, Padmavathi) and *Triticum aestivum* var. local. The test plants at 3–4 leaf stage were sap inoculated and observed for development of symptoms for 2–4 weeks. Inoculated and subsequently developed leaves were tested by DAC-ELISA and back-inoculated on to healthy sorghum cv. Rio plants to confirm the virus infections.

The antigenic relationships between virus isolates from Tirupati (SCSMV-AP) and the three other isolates were determined by employing agar gel double diffusion (AGDD), direct antigen coating-enzyme-linked immunosorbent assay (DAC-ELISA) and electroblot immunoassay (EBIA) tests.

AGDDT was performed as described by Purcifull and Batchelor[37]. The gel medium (0.8%) was prepared by using agarose melted in PBS (0.01 M potassium phosphate buffer, pH 7.0 + 0.85% sodium chloride + 0.5% SDS). The infected sorghum leaf antigens of three isolates along with SCSMV-AP isolate and healthy leaf antigens were extracted as described in the purification protocol up to high speed pelleting[36]. The pellets were suspended in minimal volume of resuspension buffer (0.02 M HEPES, pH 7.2). The peripheral wells were filled with 30 µl of partially purified preparations of the four isolates and the central well filled with 30 µl of crude antiserum of SCSMV-AP isolate. Healthy leaf and buffer controls were included and kept in the moist chamber at 37°C for 24 h. DAC-ELISA was performed as described by Hobbs *et al.*[38]. The plates were coated with leaf antigens of healthy sorghum and virus-infected sorghum isolates prepared in carbonate buffer, pH 9.6 (1 g/4.5 ml). Polyclonal antiserum of SCSMV-AP isolate produced previously was used at 1 : 500 dilution in PBS-TPO (200 µl/well)[30]. Alkaline phosphatase labelled-goat anti-rabbit antibodies (Genei, Bangalore) were used at 1 : 1000 dilution. The plate with substrate (5 mg/10 ml buffer, 200 µl/well) was incubated in dark at room temperature for 90 min and absorbance at 405 nm measured in Bio-Tek Ceres 900 ELISA plate reader.

EBIA was conducted as described by Burgermeister and Koenig[39]. For extraction of total leaf proteins, 500 mg of healthy and virus-infected leaf samples were frozen in liquid nitrogen, macerated by adding 0.1 M Tris buffer, pH 8.0 and centrifuged at 8,000 rpm for 10 min. Supernatants were loaded (100 µl/well) on to a 12% SDS-polyacrylamide gel and electrophoresed[40]. The resolved proteins from the gel were electroblotted on to nitrocellulose membrane using semi-dry blot apparatus (Novablot). The blots were blocked with 5% milk powder in TBS (20 mM Tris, 500 mM NaCl; pH 7.5) for 2 h at room temperature. The antiserum of SCSMV-AP at 1 : 500 dilution and peroxidase labelled-goat antirabbit antibodies at 1 : 1000 dilution were used in the subsequent steps. Finally, the membrane was treated with a substrate solution containing 0.05 M sodium citrate, pH 5.2, 0.03% $H_2O_2$ and 1% diaminobenzidine. Colour development was recorded and stopped by washing the membrane in distilled water.

The cDNA clone (pSV-7) with insert size of 495 bp specific to SCSMV-AP viral RNA was released by *Bam*HI/*Hin*dIII digestion[36] and the fragment was eluted from low melting agarose gel (1%)[41]. The cDNA insert (100 ng) was labelled by following random primer labelling method of Feinberg and Vogelstein[42] using $\alpha$-$^{32}$P dATP (Amersham International). Reaction was monitored to assess the per cent incorporation and free label was removed by Sephadex G-50 spun column chromatography[41].

Total nucleic acids were extracted from 50 mg each of healthy and virus-infected sorghum leaf samples according to the procedure of Smith *et al.*[43]. Total nucleic acid extracts from leaf samples infected with four isolates were made up to 200 µl with 2 × SSC. The Nylon membrane (Boehringer Mannheim) equilibrated in water followed by 10 × SSC was fitted in slot-blot filtration

manifold (Hybri Slot Manifold – GIBCO BRL). The test samples along with controls were denatured in boiling water for 5 min and chilled quickly in ice-bath and loaded on to the membrane with gentle vacuum. The membrane was air dried, baked at 80°C for 2 h and used for hybridization.

Nylon membrane was prehybridized in prehybridization solution containing 6 × SSC (from 20 × stock), 5 × Denhardt's solution (from 50 × stock), 0.5% SDS and 100 µg/ml heat denatured salmon sperm DNA (5 ml/100 $cm^2$ filter) in a hybridization bottle (Amersham) at 65°C for 4 h. DNA probe was denatured in a boiling waterbath for 5 min followed by rapid chilling on ice. Denatured probe ($10^7$ cpm/ml) was added to nylon membrane containing fresh prehybridization solution (3 ml/100 $cm^2$ filter). The membrane was hybridized at 65°C for 16 h and washed in 2 × SSC containing 0.1% SDS for 15 min at room temperature, followed by two washes at 65°C for 15 min each time in 1 × SSC, 0.5 × SSC containing 0.1% SDS. The detection was carried out by exposing the membrane to an X-ray film (Kodak) at –70°C overnight with an intensifying screen (Amersham) and later developed.

In the present study, the four virus isolates collected from different states in South India were mechanically transmitted to *Sorghum bicolor* cv. Rio plants. After 15 days of sap inoculation, the sorghum plants exhibited chlorotic or yellowish stripes alternating with normal green portions of the leaves giving mosaic pattern. Mosaic symptoms in the form of discontinuous chlorotic streaks and stripes were noticed on vegetatively propagated sugarcane.

The 11 sorghum differentials tested reacted to sap inoculation with the four isolates with varied symptoms and per cent infection (Table 1). The three isolates, like SCSMV-AP isolate, infected several hosts but the reactions of *Sorghum almum*, *S. halepense* and *S. controversum* are different with a range of 10 to 40% infection, indicating slight variability in the virulence of the virus isolates. Infection of *S. halepense* suggests that these virus isolates are not related to SCMV[14]. On the cv. Atlas, these isolates induced mosaic symptoms without necrosis, unlike the strains of the four potyviruses of the SCMV subgroup tested by Tosic *et al.*[44]. Like SCSMV-AP, the three isolates failed to infect *P. typhoides*, *E. coracana*, *Z. mays* and *T. aestivum*. Rao *et al.*[35] compared biological properties of 16 isolates of SCMV/MDMV in India and revealed that some of these isolates could infect *Z. mays* along with many sorghum differentials. Representative symptomatic leaves reacted positively in DAC-ELISA with SCSMV-AP antiserum and in bioassay tests whereas leaf samples from symptomless plants did not react in both the tests.

**Table 1.** Reaction of sorghum differentials to virus isolates causing mosaic disease of sugarcane in South India

| Host plants | Isolates | | | |
|---|---|---|---|---|
| | Tirupati isolate (Chittoor dist., AP) (SCSMV-AP) | Tanuku isolate (W. Godavari dist., AP) | Hospet isolate (Bellary dist., Karnataka) | Coimbatore isolate (Tamil Nadu) |
| *Sorghum almum* (S.88) | NS, MM, CS (27.3%) | MM, CS (20.0%) | MM, CS (37.5%) | NS, MM, CS (27.8%) |
| *S. caudatum* (IS.12730) | SM, CCSt (100%) | SM, CCSt (100%) | SM, CCSt (100%) | SM, CCSt (100%) |
| *S. cernuum* (IS.1054) | MoM, CSp (100%) | MoM, CSp (100%) | MoM, CSp (100%) | MoM, CSp (100%) |
| *S. nigricans* (IS.8887) | SM, CCSt (100%) | SM, CCSt (66.6%) | SM, CCSt (70%) | SM, CCSt (100%) |
| *S. verticilliflorum* (S.1) | MoM, CSp (100%) | MoM, CSp (100%) | MoM, CSp (100%) | MoM, CSp (100%) |
| *S. halepense* (S.77) | MoM, CS (14.1%) | MoM, CS (25%) | MoM, CS, PDL (25%) | MoM, CS, PDL (25%) |
| *S. saccharum* (IS.2866) | MoM, CSp (100%) | MoM, CSp (100%) | MoM, CSp (100%) | MoM, CSp (100%) |
| *S. controversum* (S.189) | MM (22.2%) | MM, CSp (16.1%) | – | MM, CS, PDL (27.3%) |
| *S. bicolor* cv. Atlas (IS.671) | SM, CCSt (68.2%) | SM, CCSt (80%) | SM, CCSt (58%) | SM, CCSt (80%) |
| *S. bicolor* cv. Collier (IS.649) | SM, CCSt (100%) | SM, CCSt (100%) | SM, CCSt (100%) | SM, CCSt (100%) |
| *S. bicolor* cv. Sart (IS.685) | SM, CCSt (100%) | SM, CCSt (100%) | SM, CCSt (100%) | SM, CCSt (100%) |

CCSt, Continuous chlorotic streaks; CS, Chlorotic spots; CSp, Chlorotic stripes; NS, Necrotic streaks; MM, Mild mosaic; MoM, Moderate mosaic; SM, Severe mosaic; and PDL, Purple discoloration of leaves.
Figures in parentheses indicate per cent infection based on visual observation of symptoms.

The fusion of precipitin lines in AGDDT showed that the four isolates were antigenically similar (Figure 1) and it was further confirmed by more sensitive methods like DAC-ELISA and EBIA analysis (Table 2, Figure 2). No reaction was noticed with healthy antigen. In the previous study, SCSMV-AP was found to react with polyclonal antisera of uncharacterized flexuous filamentous virus isolates causing mosaic disease of sorghum in Maharashtra[30] and sugarcane in Uttar Pradesh (data not shown). Later, it was also reported to react weakly with antiserum of characterized narcissus latent virus (NLV) from UK[36]. But it failed to react with antisera of several potyviruses including potyvirus group specific antiserum[30,36]. This clearly demonstrated that the mosaic disease of sugarcane in India is not caused by the strains of SCMV subgroup of viruses.

A more precise approach to discriminate viruses and related strains is to use defined nucleic acid probes made to specific regions of the genomic RNA, including the 3′-untranslated region (3′-UTR). In the family Potyviridae, the 3′-UTR of viral RNA has great value in the identification of potyviruses and can be used to distinguish viruses from strains[45,46]. The genomic relationship of South Indian virus isolates with SCSMV-AP was determined using the clone pSV-7 representing 3′-UTR and C-terminal part of the coat protein gene of SCSMV-AP in hybridization studies. The total nucleic acid extracts of leaf samples infected by these isolates showed positive signal with $^{32}P$ probe (Figure 3), indicating that they are related to SCSMV-AP at the genome level. No reaction was noticed with healthy sorghum leaf samples. The 3′-UTR differs considerably in length and sequence between distinct potyviruses, but is very similar in length and sequence in related strains of the same virus[45,47]. Nucleic acid hybridization involving the 3′-UTR of the potyviral genome was used to support the proposal that BYMV was distinct from CYVV[48], WMV-2 and SMV-N were strains of the same virus[49] and to confirm that MDMV-B was a strain of SCMV[50]. Earlier data based on random cDNA hybridization indicated that pea mosaic virus was a strain of BYMV, but by using nucleic acid hybridization involving the 3′-UTR it was identified that pea mosaic virus was distinct from BYMV[51].

We conclude that the three isolates from South India are antigenically similar to SCSMV-AP and it was further

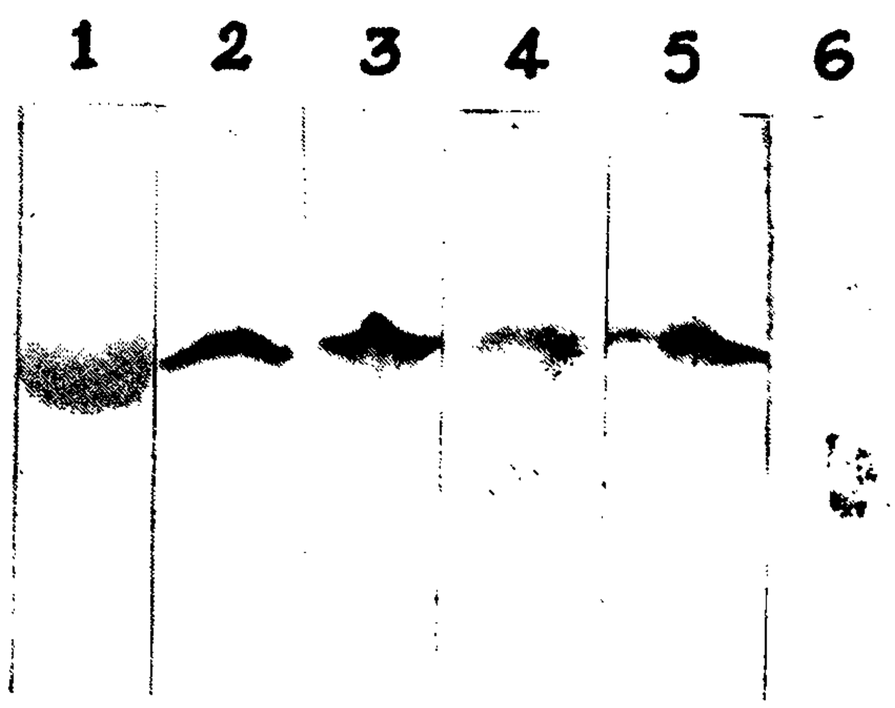


**Figure 2.** EBIA analysis of South Indian sugarcane virus isolates probed with the antiserum of Tirupati isolate from Chittoor district. Lane 1, Disrupted Tirupati isolate coat protein; Crude sorghum leaf extracts; lane 2, Tirupati isolate (Chittoor district, AP) (SCSMV-AP); lane 3, Tanuku isolate (West Godavari district, AP); lane 4, Coimbatore isolate (Tamil Nadu); lane 5, Hospet isolate (Bellary district, Karnataka); and lane 6, Healthy sorghum leaf.

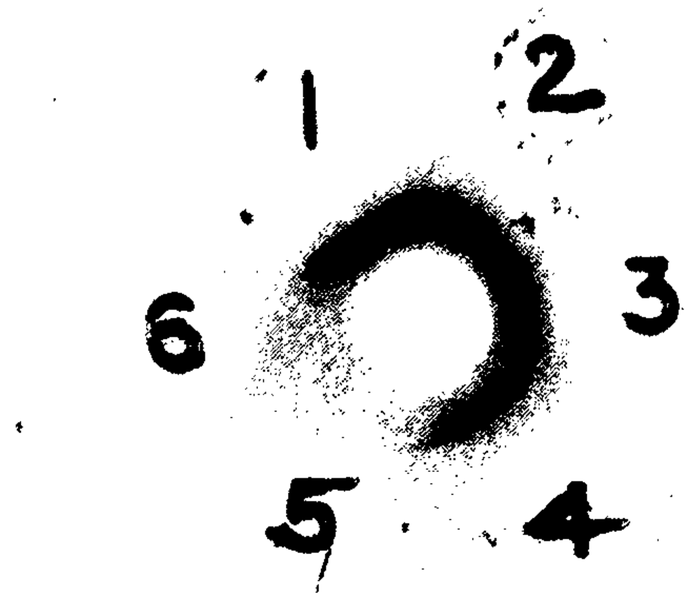


**Figure 1.** AGDD analysis of South Indian sugarcane virus isolates probed with the antiserum of Tirupati isolate from Chittoor district in the central well. The peripheral wells contained partially purified virus preparations. 1, Tirupati isolate (Chittoor district, AP) (SCSMV-AP); 2, Tanuku isolate (West Godavari district, AP); 3, Coimbatore isolate (Tamil Nadu); 4, Hospet isolate (Bellary district, Karnataka); 5, Healthy sorghum leaf; and 6, Buffer control.

**Table 2.** Reaction of South Indian sugarcane virus isolates with SCSMV-AP antiserum in DAC-ELISA

| Antigen samples | $A_{405}$ nm |
|---|---|
| Tirupati isolate (Chittoor district, AP) (SCSMV-AP) | 2.98* |
| Tanuku isolate (West Godavari district, AP) | 2.72 |
| Hospet isolate (Bellary district, Karnataka) | 2.89 |
| Coimbatore isolate (Tamil Nadu) | 2.65 |
| Healthy sorghum | 0.23 |

*Values represent an average of $A_{405}$ nm readings (in triplicate).

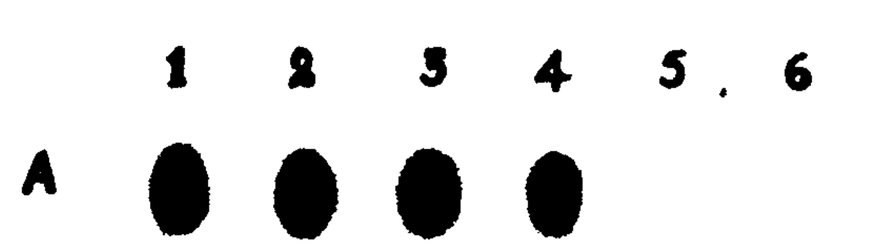


**Figure 3.** Slot-blot hybridization analysis of South Indian sugarcane virus isolates probed with $^{32}P$-labelled pSV-7 insert of the Tirupati isolate from Chittoor district. Lane 1, Tirupati isolate (Chittoor district, AP) (SCSMV-AP); lane 2, Tanuku isolate (West Godavari district, AP); lane 3, Coimbatore isolate (Tamil Nadu); lane 4, Hospet isolate (Bellary district, Karnataka); lane 5, Healthy sorghum leaf; and lane 6, Buffer control.

confirmed by genome-based approach. However, they differed in inciting reactions on certain sorghum differentials. Thus the present study establishes that the mosaic disease of sugarcane in South India is caused by pathotypes of SCSMV-AP.


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