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Peanut green mosaic virus – a member of the potato virus Y group infecting groundnut (*Arachis hypogaea*) in India

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SUMMARY

A virus, now named peanut green mosaic virus (PGMV), was isolated from groundnut (*Arachis hypogaea*) in India and identified as a member of the potato virus Y group by electron microscopy, aphid transmission, and its chemical properties. It was sap transmissible to 16 species of the Leguminosae, Solanaceae, Chenopodiaceae, Aizoaceae and Pedaliaceae; *Phaseolus vulgaris* was a good local lesion host. PGMV remained infective in buffered groundnut leaf sap at dilutions of 10^{-3} after 3 to 4 days at 25 °C, or heating for 10 min to 55 °C but not 60 °C. PGMV was transmitted in the non-persistent manner by *Aphis gossypii* and *Myzus persicae* but was not seed-borne.

Purified virus preparations contained flexuous filamentous particles *c.* 750 nm long which sedimented as a single component with a sedimentation coefficient (S°_{20w}) of 171S, and contained a single polypeptide (mol. wt 34 500 daltons) and one nucleic acid species (mol. wt $3{\cdot}25 \times 10^6$ daltons). PGMV is serologically unrelated to peanut mottle virus (PMV) and other viruses infecting leguminous crops. Infected leaves contained cylindrical, cytoplasmic inclusions.

INTRODUCTION

A disease characterised by mosaic mottling and vein clearing of young leaves was observed in 1970 in crops of groundnut (*Arachis hypogaea*) in the Chittoor district of Andhra Pradesh, India. These symptoms differed from those induced by any of the viruses previously reported from groundnut in India. Although many virus diseases of groundnut have been described in India (Chohan, 1974; Raychaudhuri, 1977) only those caused by peanut mottle virus (PMV) and tomato spotted wilt virus (TSWV) (Reddy *et al.*, 1978; Ghanekar *et al.*, 1979) have been fully investigated. We report here the symptomatology, host range, transmission, physical and chemical properties, and serological affinities of the virus from Chittoor which we have named peanut green mosaic virus (PGMV).

MATERIALS AND METHODS

Culture and maintenance: The virus was first established in a screenhouse by graft-inoculating healthy groundnut seedlings (cv. TMV 2) from which it was then mechanically transmitted to further groundnut plants and to French bean (*Phaseolus vulgaris*). The virus isolated from a single local lesion produced on French bean, was maintained in groundnut.

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All sap inoculations were made on Carborundum-dusted leaves with a cloth pad dipped in sap extracted in 0·05 M phosphate buffer, pH 7·0, containing 0·02 M 2-mercaptoethanol (PBM).

Assay host. Since French bean (cv. Local) consistently produced chlorotic local lesions, it was used as the assay host. Fully expanded primary leaves were chosen for inoculation and all assays used at least eight half leaves for each treatment.

Host range. In host range studies, six plants of each test species or cultivar were inoculated. Back tests of inoculated and uninoculated leaves, were made to French bean and groundnut.

Properties in sap. The physical properties of the virus were studied in crude groundnut leaf extracts diluted to 10^{-1} in 0·01 M phosphate buffer, pH 7·0. To determine the thermal inactivation point 1 ml of sap was heated in a water bath for 10 min at various temperatures.

Seed transmission. Seeds from mechanically inoculated groundnut (cv. TMV-2) with conspicuous symptoms were sown in sterile soil and kept in a screenhouse. Seedlings about 2 wk old were tested for virus by inoculating French bean.

Aphid transmission. In transmission studies *Aphis craccivora, A. gossypii* and *Myzus persicae* colonies were maintained on healthy pigeonpea, eggplant, and cauliflower respectively. The aphids were starved for *c.* 1 h and then allowed to make a single acquisition probe of 30 s to 3 min on detached, young, infected groundnut leaves. At least 10 insects were then transferred to each healthy groundnut test plant and after 14 h, killed by spraying with 0·025% oxydemeton-methyl (Bayer, India). Test plants showing symptoms were assayed on French bean.

Virus purification. Infected groundnut leaves were homogenised, clarified with chloroform and the virus precipitated by adding 0·2 M NaCl and 4% (w/v) polyethylene glycol. Further purification was by rate and quasi-equilibrium zonal density gradient centrifugation in sucrose (Iizuka, Rajeshwari & Reddy, unpublished data). The virus from the single light scattering zone produced by quasi-equilibrium zonal density gradient centrifugation was pelleted at 36 000 rev./min for 1 h in a Beckman R-40 rotor. The virus pellet, suspended in 0·01 M borate-phosphate buffer (pH 8·3) containing 0·2 M urea (BPU), was used for electron microscopy, infectivity tests and studies of physical and chemical properties.

Sedimentation coefficient. The sedimentation coefficient of the purified virus was determined in a Beckman Model E analytical ultracentrifuge with an An-D rotor using Schlieren optics. After attaining a speed of 21 000 rev./min photographs were taken at 2 min intervals.

Electron microscopy. Purified virus preparations were stained in neutral 2% sodium phosphotungstate and observed in a Hitachi model H-300 electron microscope. Thin sections were cut of healthy and infected groundnut and *Nicotiana benthamiana* leaves. Tissues were first fixed in 3·5% glutaraldehyde, in 0·1 M phosphate buffer, pH 7·2, for 2 h at 4 °C, then post-fixed for 2 h at 4 °C in 1% osmium tetroxide in the same buffer. Dehydration was in acetone and embedding in Epon 812. Sections, cut with glass knives, were stained with uranyl acetate and lead citrate before examination.

Polyacrylamide gel electrophoresis. Purified virus pellets were solubilised in 6 M urea in 0·0625 M Tris-HCl buffer (pH 6·8) containing 20 g/l sodium dodecyl sulphate (SDS) and 1% (v/v) 2-mercaptoethanol. The mol. wt of the polypeptide was determined by the method of Laemmli (1970), as modified by Reddy & Black (1977). The resolving gel contained 10% acrylamide and 0·27% bisacrylamide prepared in 0·375 M Tris-HCl buffer (pH 8·8) with 1 g/l SDS and 0·5 M urea. A spacer gel (0·6 × 2·0 cm) of 3·6% acrylamide and 0·09% bisacrylamide in 0·125 M Tris-HCl buffer (pH 6·8) containing 1 g/l SDS and 0·5 M urea, was layered on the resolving gel. The solubilised virus samples were co-electrophoresed with markers at 30 V for 6 h. The gels were stained as described by Reddy & MacLeod (1976) and scanned at 540 nm in a Gilford recording spectrophotometer. The markers used to estimate the mol. wt of the PGMV polypeptide were phosphorylase B, bovine serum albumin, egg albumin, chymotrypsinogen, soybean trypsin inhibitor and lysozyme (Bio-rad Laboratories).

Nucleic acid was obtained from purified PGMV as described by Kirby (1965) with a few modifications. Purified virus was suspended in 1 × SSC (0·15 M sodium chloride, 0·015 M sodium citrate, pH 7·4). Sodium dodecyl sulphate (100 g/l in 1 × SSC) was added to give a concentration of 1% before being treated with Kirby's phenol mixture. The whole mixture was heated at 60 °C for 2 min. Phenol in the aqueous phase was removed by five extractions with ether; ether was evaporated by exposing to air and continuously agitating. The nucleic acid obtained was re-extracted in phenol and finally precipitated by adding 2·5 volumes of absolute ethanol. 3 M sodium acetate (pH 5·5) was added to give 0·2 M in the final volume. Following overnight precipitation at −13 °C the precipitate was washed twice with ethanol and finally resuspended in 0·1 × SSC containing 50 g/l sucrose.

Nucleic acid was also obtained by treating purified PGMV with pronase, SDS, 2-mercaptoethanol and urea. Purified virus suspended in 0·1 × SSC was treated with pre-incubated pronase (1 mg/ml) at 37 °C for 2 h followed by SDS to give a final concentration of 10 g/l, 2-mercaptoethanol to give a concentration of 2 g/l and urea to 0·1 M. The mixture was incubated at 37 °C for 2 h and before loading on gels sucrose was added to give 50 g/l.

Nucleic acid analysis was by the method of Loening (1967), as described by Adesnik (1970). Nucleic acid samples were electrophoresed in cylindrical gels (0·6 × 8 cm) containing 2·4% acrylamide, 0·12% bisacrylamide and 0·5% agarose. The buffer for electrophoresis was 0·04 M Tris, containing 0·02 M sodium acetate and 0·002 M ethylenediaminetetraacetate (disodium salt), pH 7·6. The nucleic acid and marker samples were electrophoresed at 50 V for 3 h at room temperature. Gels were then suspended for 20 min in 0·4 M acetate buffer, pH 4·7, and stained in 0·1% toluidene blue O for 10 min; gels were destained in several changes of distilled water and scanned at 540 nm. The mol. wt of the PGMV nucleic acid was estimated using tobacco mosaic virus RNA ($2·0 \times 10^6$) and *Escherichia coli* 23 S ($1·07 \times 10^6$) and 16 S ($0·55 \times 10^6$) ribosomal RNA as markers.

Serology. New Zealand White inbred rabbits were given three intramuscular injections at 6 day intervals followed by two intravenous injections at 7 day intervals. For each intramuscular injection 1 mg of purified virus, suspended in 0·5 ml of 0·01 M borate, phosphate buffer, pH 8·3, was emulsified with 0·5 ml Freund's incomplete adjuvant. Serum was collected 3 wk after the final intravenous injection.

Antiserum titres were determined by the ring interface precipitin test and serological relationships tested by the Ouchterlony gel double diffusion test (Ball, 1974) in 0·8% (w/v) agarose in 0·01 M phosphate buffer (pH 7·0) containing 0·85% (w/v) NaCl, 0·5% (w/v) sodium azide, and 0·5% (w/v) 3,5-diiodosalycilic acid. All plates, after adding the reagents, were incubated at room temperature in a humid chamber for 3 to 7 days.

Haemagglutination tests were similar to those described by Reddy *et al.* (1978). Tanned sheep red blood cells at 25 g/l were coated with antibody solution diluted to 1/50 in 0·85% (w/v) NaCl, at 37 °C for 30 min. Excess antibody was removed by low speed centrifugation and the cells washed twice in 0·15 M phosphate buffered saline, pH 7·2, (PBS) and resuspended in PBS containing 0·5% (w/v) bovine serum albumin (BSA-PBS) to give a concentration of 25 g/l packed cells. Extracts of test plants in PBM were clarified in a refrigerated centrifuge for 10 min at 5000 *g*. Serial two fold dilutions of the supernatants were prepared in BSA-PBS and 0·5 ml was put in each well; 0·08 ml of sensitised cells was added to each well and gently mixed. The plate was left at room temperature for *c.* 1 h and then incubated overnight at 4–6 °C. A positive haemagglutination reaction was a definite matting of cells with irregularity of the peripheral ring: in a negative reaction a peripheral ring of cells encircled a central pale disc.

RESULTS

Symptomatology. Mechanically inoculated groundnut plants showed chlorotic spots and vein clearing on young, systemically infected quadrifoliate leaves 12 to 14 days after inoculation, later

leaves showed a severe mosaic (Plate, fig. 1). As infected leaves aged the symptoms become less obvious. Infected plants were stunted.

Assay host. Chlorotic or necrotic local lesions were produced on the primary leaves of three bean cultivars. French bean, cv. Local, developed chlorotic lesions on inoculated leaves 6 to 8 days after inoculation; later the lesions became necrotic and some spread along the veins. Interveinal tissue turned yellow (Plate, fig. 2).

Host range. The virus also induced chlorotic or necrotic local lesions in *Cassia obtusifolia*, (Plate, fig. 3), *Chenopodium amaranticolor*, *C. quinoa*, *Cymopsis tetragonoloba*, *Phaseolus vulgaris* (cvs Bountiful & Kintoki) and *Tetragonia expansa*; *Cymopsis tetragonoloba* and *P. vulgaris* (cv. Kintoki) were also systemically infected. Systemic mosaic symptoms were also induced in *Cassia occidentalis*, *Nicotiana clevelandii*, *N. benthamiana* (Plate, fig. 4), *N. glutinosa* × *N. clevelandii* and *Sesamum indicum*. The virus failed to infect *Beta vulgaris*, *Brassica oleracea*, *Cajanus cajan*, *Canavalia ensiformis*, *Capsicum annuum*, *Crotalaria juncea*, *Cucumis sativus*, *Datura stramonium*, *Glycine max* (cv. Bragg), *Gomphrena globosa*, *Lycopersicon esculentum*, *N. rustica*, *N. glutinosa*, *N. tabacum* (cv. Samsun), *Ocimum basilicum*, *Phaseolus aureus*, *P. mungo*, *P. limensis*, *P. vulgaris* (cvs Kentucky Wonder Waxpole, Idaho Refugee, Pinto U.I. No. 72 and Topcrop), *Physalis floridana*, *Pisum sativum* (cv. Bonneville), *Raphanus sativus*, *Sesbania grandiflora*, *Solanum melongena*, *Vicia faba*, *Vigna unguiculata* (cvs C-152 and early Ramshorn) and *Zinnia elegans*.

Properties of the virus in buffered sap. The thermal inactivation point was between 55° and 60 °C, the infectivity dilution end point between 10^{-3} and 10^{-4} and infectivity was retained for 3 to 4 days at 25 °C.

Seed transmission. Of the 480 seeds collected from mechanically infected plants 460 germinated, none showed PGMV symptoms and no virus could be isolated from seedlings.

Aphid transmission. After a 30 s acquisition access period both *A. gossypii* and *M. persicae* transmitted the virus to 26 of 50 groundnut and three of five *N. benthamiana* plants. None of the 20 groundnut plants and three *N. benthamiana* plants exposed to aphids from healthy colonies was infected. *A. craccivora* collected from pigeonpea and groundnut in the Tirupati and Hyderabad areas, was unable to transmit the virus irrespective of the length of acquisition feeds.

Properties of purified virus. (a) Ultraviolet absorption spectrum. A single light scattering zone about 2·5 cm from the bottom of the tube was produced by equilibrium zonal density gradient centrifugation. Samples from this zone diluted in PBM produced typical symptoms of PGMV on groundnut and French bean. The u.v. absorption spectrum of the purified virus showed a shoulder at 290 nm, and the E 260/280 ratio was 1·19 indicating that the virus contained about 5% nucleic acid (Gibbs & Harrison, 1976). The *A* 260/245 ratio was 1·26. Assuming an extinction coefficient of 3·0, nearly 1 mg of virus was obtained from 50 g of leaf.

(b) Electron microscopy. Purified virus preparations contained flexuous filamentous rods *c.* 750 nm long (Plate, fig. 5). Thin sections of infected groundnut and *N. benthamiana* leaves contained cylindrical cytoplasmic inclusions (Plate, fig. 6).

(c) Sedimentation coefficient. PGMV suspended in 0·01 M borate-phosphate buffer (pH 8·3) sedimented as a single component with a sedimentation coefficient (S°_{20w}) of 171 *S* (Plate, fig. 7).

(d) Polyacrylamide gel analysis of protein and nucleic acid. When disrupted with SDS, urea and mercaptoethanol, PGMV formed one band following electrophoresis in 10% acrylamide gels (Plate, fig. 8). The mol. wt of the PGMV polypeptide was estimated to be 34 500 daltons.

Nucleic acid prepared by both methods migrated as a single band electrophoresed in 2·4% acrylamide gels (Plate, fig. 9), and its mol. wt was estimated to be $3{\cdot}25 \times 10^6$ daltons; when co-electrophoresed the RNAs migrated as a single band.

Serology. The titre of the PGMV antiserum as determined by ring interface tests was 1/256. In agar gel diffusion tests purified virus consistently gave a single precipitin line; undiluted

PGMV antiserum did not react with undiluted healthy groundnut leaf extracts. No precipitin lines were observed when purified PGMV was tested against the antisera of clover yellow vein, soybean mosaic, adzuki bean mosaic, bean yellow mosaic and peanut mottle viruses.

In haemagglutination tests, extracts from healthy and peanut mottle virus (PMV)-infected groundnut leaves gave no reaction but PGMV-infected leaf extracts had a titre of 1/6400 to 1/12800. In addition, cells sensitised with PMV antiserum gave no reaction when tested with PGMV-infected leaf extracts and purified PGMV preparations.

DISCUSSION

The size and morphology of PGMV particles, the presence of cylindrical inclusion bodies in infected cells, the sedimentation coefficient and mol.wt of its coat protein sub-unit and nucleic acid, its aphid transmission and properties in crude sap place PGMV in the potyvirus group. Peanut mottle virus has also been shown to be a potyvirus occurring naturally in groundnut (Kuhn, 1965; Herold & Munz, 1969; Bock & Kuhn, 1975). PGMV differs from PMV in host range and serology and is mechanically transmissible to *Petunia hybrida*, *Chenopodium amaranticolor* and *C. quinoa* whereas PMV does not infect these hosts. However, PGMV failed to infect *Phaseolus vulgaris* (cv. Topcrop) which is an important local lesion host for PMV. Also PGMV does not infect *Pisum sativum*, *Canavalia ensiformis*, *Glycine max*, *Phaseolus vulgaris* (cvs Kentucky Wonder Waxpole and Pinto U.I. No. 72) which are hosts of PMV. Unlike PMV, PGMV was not seed transmitted.

The virus (PESV) causing peanut eye spot (Dubern & Dollet, 1978) was recently shown to be a potyvirus. PESV, however, differs from PGMV in failing to infect *Chenopodium amaranticolor* and *Cassia occidentalis*. In addition PGMV does not infect *Glycine max* and *Pisum sativum*, which are hosts of PESV. In Japan turnip mosaic virus (TuMV) was also isolated from groundnut plants (Inouye & Inouye 1964) but PGMV differs from TuMV in failing to infect *Brassica oleracea*, *Gomphrena globosa* and *Raphanus sativus*. In laboratory tests, groundnut was infected with bean yellow mosaic, pea mosaic and passionfruit woodiness viruses of the potyvirus group (Edwardson, 1974). Although more serological tests are needed to determine the serological relationship of PGMV with other potyviruses, PGMV is considered to be a distinct and previously undescribed virus.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Typical mosaic symptoms on groundnut leaves induced by PGMV.

Fig. 2. Necrotic local lesions in primary leaves of *P. vulgaris* (French bean cv. Local) induced by PGMV.

Fig. 3. Necrotic local lesions on *C. obtusifolia* induced by PGMV.

Fig. 4. Systemic mosaic symptoms induced by PGMV on *N. benthamiana*.

PLATE 2

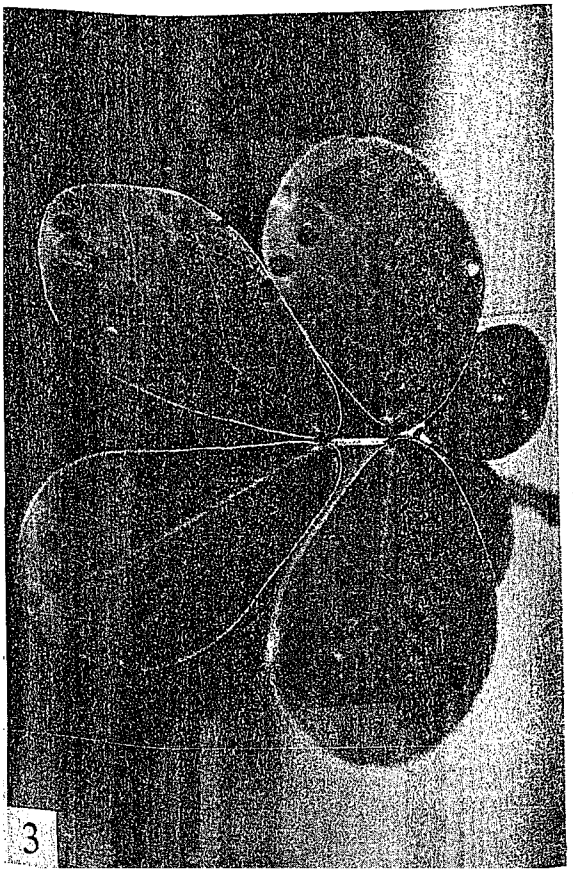
Fig. 5. Negatively stained PGMV particles purified from groundnut leaves. Bar represents 250 nm.

Fig. 6. Electron micrograph of a thin section of a leaf of PGMV-infected groundnut showing circular inclusions (shown by arrows). Bar represents 200 nm.

Fig. 7. Schlieren picture of a purified PGMV preparation after 8 min at 21 000 rev/min.

Fig. 8. Polyacrylamide gel profiles of marker proteins + PGMV polypeptide (A) and PGMV polypeptide (B). Movement was from top to bottom. Electrophoresis was for 6 h at 30 V.

Fig. 9. Polyacrylamide gel profiles of PGMV RNA extracted by Kirby's method (A), PGMV treated with pronase, SDS, 2-mercaptoethanol and urea (B) and marker RNAs (C). Movement was from top to bottom. Electrophoresis was for 3 h at 50 V.



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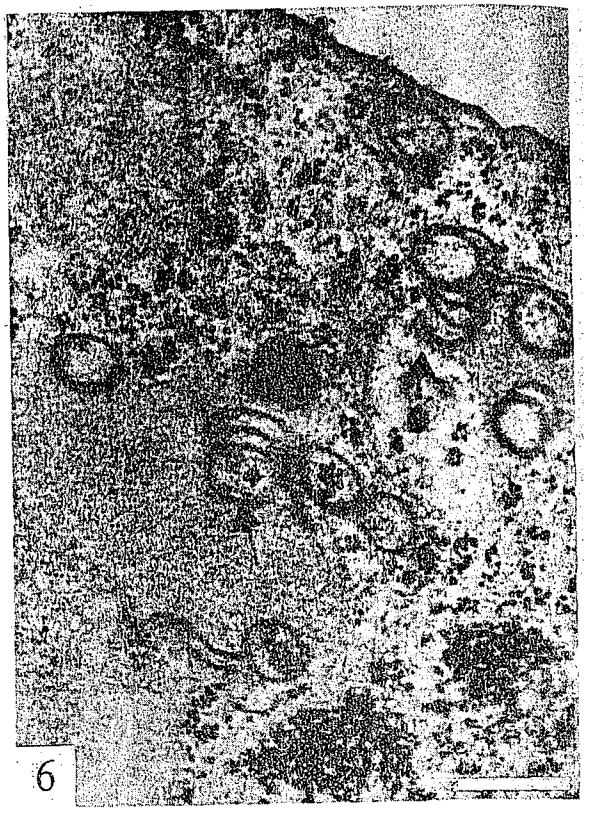

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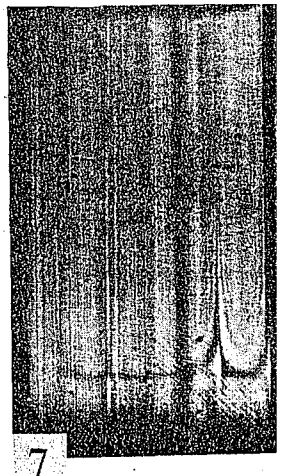

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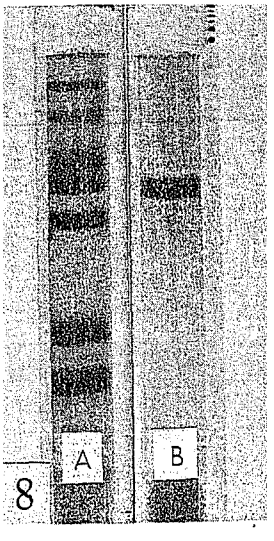

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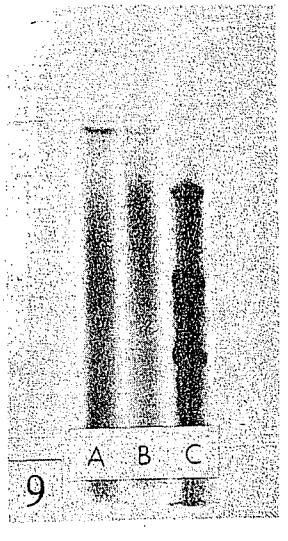

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