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Evidence of true seed transmissible nature of turnip mosaic virus in mustard species

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Abstract

Mustard is a commercial oilseed crop worldwide infected by a highly infectious turnip mosaic virus (TuMV). In the experimental field at ICAR-IARI, New Delhi, in 2022, a 100% incidence of TuMV infection was observed in brown, black and yellow mustard. A very low aphid population suggested the possibility of seed transmission. Earlier, the virus genome was characterized by high throughput sequencing and it was a recombinant of World-B and Asian-BR isolates. The presence of TuMV in immature seeds was confirmed in eight field-grown genotypes via RT-PCR using CP-specific primers designed from the same genome sequence. TuMV was found to be localized in embryo and cotyledon, indicating its true seed-borne nature. Presence of TuMV was also confirmed by RT-PCR in the grow out plants from seeds of field grown eight infected genotypes and 9 genotypes collected from seed stock, that were grown in an aphid-free growth chamber. Further, out of 24 seedlings of Pusa Gold (seed stock) and Pusa Karishma (seeds from field grown plants), 20 and 17 seedlings were found infected with TuMV, respectively. The internally seed-borne nature of the virus leads to its early establishment at the seedling stage, leading to stunting and leaf-puckering symptoms in the progeny plants. This study is the first evidence of seed embryo infection and seedling transmission of TuMV of all the three species of mustard plants (brown, black and yellow mustard). Seed transmission of TuMV in mustard genotypes have implications for the seed exchange programme of mustard seeds.

Keywords Turnip mosaic virus, Mustard, Seed transmission, Embryo, Cotyledon, Grow-out test

Background

Plant pathogens, particularly viruses, can sustain their populations in host plants by spreading via seeds. The horizontal plant virus transmission from diseased to healthy plants occurs via grafting or sap as well as via vectors such as insects, nematodes, and fungi from virus-infected plants [1–3]. However, such pathways are most often restricted between neighbouring plants [4, 5]. Long-term viral perpetuation is exceptionally challenging, especially for viruses that infect annual plants, as most viruses cannot persist for any amount of time outside of host. An efficient way to get around this is by seed infection, which connects the pathogen's long-term survival to the host [6]. The infected seeds can be a significant route of transmission of viruses from one season to the next and from parents to offspring [7–9]. Due to the fact that most plant viruses are spread secondarily by insect vectors, even a small number of initially infected plants can cause devastating epidemics [10].

Mustard (yellow mustard, *Brassica rapa* subsp *trilocularis;* brown mustard, *B. juncea*; and also black mustard, *B. nigra*) is an important commercial crop worldwide, with its seeds serving as a condiment as well as a source of oil and leaves as vegetables. Mustard is one of the most frequently used and well-known spices and condiments in the world [11]. Several viruses belonging to different genera have been testified to infect and cause different levels of losses in

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mustard production [12]. The mosaic disease of brassica crops dates back to as old as 1921, being reported to be caused by turnip mosaic virus (TuMV) from Indiana in turnip, from Denmark in turnip and eventually from Chinese cabbage and other crucifers [13–16]. Moreover, the mosaic disease has also been reported to be caused by mixed TuMV infections and the cucumber mosaic virus (CMV) [17]. The losses inflicted by turnip mosaic on turnip in Germany ranged from 1 to 90% [18]. Further, it was projected that the rapeseed (*B. napus* var. *napus*) mosaic-causing viruses in China reduced yields by 50–80 per cent [19]. Estimates of the yield reductions in *rapeseed* plants with severe TuMV virus symptoms ranged from 70 to 79% [20].

TuMV is a member of Potyviridae family (Genus Potyvirus). The modes of transmission of TuMV were reported to be mechanical and by green peach aphid *(Myzus persicae*). The earlier reports rejected possibilities of seed transmission since mustard seeds from mosaic plants yielded healthy seedlings [21]. However, tests for seed transmission by planting seeds derived from mosaic-infected rutabaga (*Brassica napus*) plants showed symptoms of mosaic [22].

In the present study, symptoms of viral infection (mosaic, puckering, and stunting) were observed in the experimental field of mustard at the Indian Council of Agricultural Research-Indian Agricultural Research Institute (ICAR-IARI), New Delhi (Fig. 1). Association of TuMV and CMV was confirmed by characterizing the genome through deep sequencing [23, 24]. However, only TuMV was found associated with the disease in the next season of mustard crops. Since the mustard plants in the field were found to be TuMV infected even in the early sown crop when aphids were absent, the possibility of seed transmission was hypothesized and tested in the present study. The study demonstrates the seed transmission of TuMV in mustard species for the first time, validating its true seed-borne as well as vertically transmissible nature to next-generation plants.

Materials and methods

Collection of seeds and RNA isolation

On observation of viral symptoms in the mustard fields exhibiting puckering and stunting symptoms, the leaf samples were collected and subjected to virome analysis via High-throughput sequencing (HTS) [24], followed by validation through reverse transcription polymerase chain reaction (RT-PCR). The plants found infected by TuMV were tagged. During 2021-22, the siliqua containing immature seeds of eight genotypes (PDZ1, PDZ5,

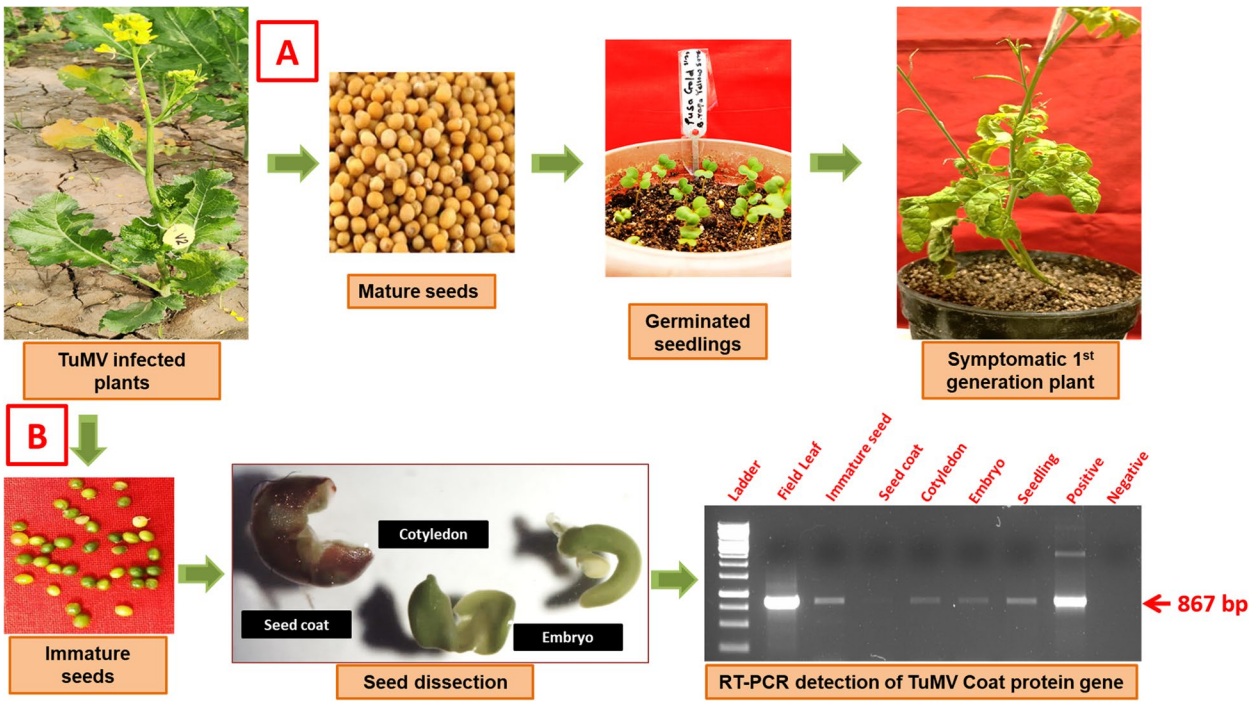

Fig. 1 Detection of TuMV infection in mustard leaves, seeds and seedlings. Mustard plants in the field showing stunting and leaf puckering. **A** Mature seeds obtained from infected plants and seedling germinated from mature seeds for grow-out test followed by symptomatic 1st generation plant grown from infected seedlings. **B** Immature mustard seeds obtained from infected plants and seed coat separated from cotyledon and embryo of immature seeds. PCR analysis performed with TuMV CP-specific primers from different organs of TuMV infected mustard plants. Ladder – 1 kb DNA ladder marker

PDZ11, Pusa Karishma, PM30, Pusa Vijay, JC-21, and JC-33) were harvested from 70 to 80 days old tagged mustard plants in IARI fields. The genotypes can be categorized as conventional (Pusa Vijay), single zero types (PM-30 and Pusa Karishma), and double zero types (JC-21, JC-33, PDZ1, PDZ-5 and PDZ-11) of brown mustard.

Ten immature seeds from each infected plant of the eight genotypes were added to a DEPC-treated mortar and ground in liquid nitrogen using a pestle, followed by homogenization in an extraction buffer. The total RNA was isolated according to the instructions mentioned in Sigma Spectrum™ Plant Total RNA kit. Using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA), the amount and quality was measured.

The mature seeds harvested from the asymptomatic as well as symptomatic eight genotypes of mustard plants grown in IARI fields in crop seasons 2020-21 and 2021-22 were also collected. In addition, mature seeds of nine other genotypes viz., PDZM31, PDZM33, Pusa Agrani, PM26, PM27, PM31, NRC-HB-101 of brown mustard, Pusa Gold of yellow mustard, BN-3 of black mustard were collected from the stock maintained in the Division of Genetics, ICAR-IARI, New Delhi.

Separation of seed parts followed by RNA isolation

Immature seeds from the infected mustard plants of two field grown genotypes viz., (Pusa Karishma and Pusa Vijay) as well as from plants grown from seed stocks (Pusa Gold and BN-3) were used for RNA isolation. The embryo and cotyledon of immature seeds were cautiously separated using a sterile needle and scalpel from the seed coat of seeds under the light microscope (Fig. 1) [25]. Ten bunches of each seed component were used for RNA isolation using the protocol described above (Sect. 2.1), followed by RT-PCR amplification described in Sect. 2.4.

Grow-out test

The grow-out test was conducted using mature seeds of all the 17 genotypes included in this study after surface sterilization by a 30-s exposure to 70% ethanol and three rinses in sterile distilled water. This was followed by sowing in plastic pots filled with sieved autoclaved soil. The pots were placed in a 15 sq. ft growth chamber available at the National Phytotron Facility (NPF), IARI, New Delhi, under controlled conditions of temperature and relative humidity to rule out cross-contamination by aphids (Fig. 1, Fig. S1). Similar experiments were repeated in isolated conditions in the containment facility of the Division of Plant Pathology, IARI, New Delhi. The containment chambers were fumigated by adding formaldehyde solution to potassium permanganate before sowing the surface sterilized seeds in sieved autoclaved soil-filled pots. Five seedlings (two-leaf stage) of each variety were harvested two weeks after planting and pooled for RNA isolation and RT-PCR amplification as per the procedure described for infected leaf samples. Three replicates were used for each variety. Further, to determine the seed transmission rate of TuMV in mustard, 90 mature seeds each of Pusa Gold (seed stock) and Pusa Karishma (TuMV-infected field plants of 2022) were sown in separate pots (15 seeds per pot) after surface sterilization. The pots were maintained in the growth chambers in the containment facility at the Division of Plant Pathology, IARI, New Delhi. The pots were covered using muslin cloth cages to rule out any contamination. The germination count was taken after two weeks, after which 24 seedlings per variety were harvested and each seedling was subjected to RNA isolation separately as per protocol described in Section 2.1.

Detection of viruses through polymerase chain reaction and sequencing

The FIREScript® RT cDNA synthesis kit by Solis Biodyne was used to synthesize the first strand of complementary DNA (cDNA) from the RNA isolated from immature seeds, embryos, cotyledon, seed coats and seedlings, which was subsequently amplified via PCR. The procedure included an initial denaturation phase at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C (TuMV), and extension at 72 °C for 1 min, followed by the final primer extension for 10 min at 72 °C. The PCR mixture contained 12.5 µl of Thermo Fisher Scientific's DreamTaq™ green PCR master mix, 1 µl of forward and reverse primers, and 500ng cDNA template. The TuMV CP primer set was designed from the full genome recovered from HTS (forward- 5` GCA GGTGAAACGCTTGATGCAGG 3` and reverse- 3` TAACCCCTTAACGCCAAGTAA 5`) of amplicon size 867 bp. Amplified fragments of the RT-PCR experiments were cloned and sequenced in duplicates at Eurofins Genomics India, Bengaluru, using the SP6 and T7 universal primer pair. The consensus sequences were deposited in GenBank and BLASTed on NCBI against the viral database for confirmation.

Results

Testing of samples and virus characterization

Based on the analysis of data obtained through HTS, the whole genome of TuMV was recovered from the infected plants. The TuMV presence was validated using CP-specific primers in eight genotypes grown in the field, and all were found to be infected by TuMV.

Virus detection in the different parts of seeds

The RNA isolated from the immature seeds obtained from the infected plants of eight genotypes when subjected to RT-PCR, depicted the amplification of the CP region of TuMV, indicating its presence in the immature seeds. Further, the RNA isolated from bunches of ten whole surface-sterilized seeds, ten embryos, and ten cotyledonary tissues of seeds of Pusa Gold, Pusa Karishma, Pusa Vijay, and BN-3 showed amplification of the CP region of TuMV. However, there was no amplification in the RNA obtained from a bunch of ten seed coats and thus considered free from TuMV infection (Fig. 1). This indicated the true seed-borne nature of TuMV in the mustard genotypes.

Testing of virus presence in the grown-out seedlings

In the grow-out test, the RNA isolated from the seedlings of the seeds obtained from infected plants of all the eight genotypes showed amplification of the CP region of TuMV in all the replicated experiments, thus confirming the transmission of TuMV from seed to seedling. In addition, the presence of TuMV was detected in the RT-PCR assay in the seedlings grown from the seeds of nine genotypes collected from the seed stocks of Division of Genetics, IARI, New Delhi. The seedlings generated from the seeds obtained from healthy plants were free from any virus infection (healthy). The immature seeds obtained from plants grown from the seeds of infected plants in growth chambers were also found to be infected by TuMV (Fig. 1). The seedlings that were left to grow into mature first-generation plants were observed to be symptomatic and exhibited viral symptoms such as leaf puckering, distortion, and stunting. On testing the leaf samples, these plants were found to be infected by TuMV. This study demonstrated that the infected mustard seeds could vertically transmit TuMV to the next-generation plants.

Of the 90 seeds sown in the pots, 61 seeds of Pusa Gold and 81 seeds of Pusa Karishma were found to be germinated. From the germinated seedlings, the RNA isolated from 24 seedlings separately of each variety when tested for the presence of TuMV, 20 Pusa Gold seedlings and 17 Pusa Karishma seedlings out of 24 were found to be infected with TuMV (Fig. 2).

Conformation of the virus through sequence homology

The amplified sequences of the CP gene of TuMV from the immature seeds of the JC-21 genotype and the

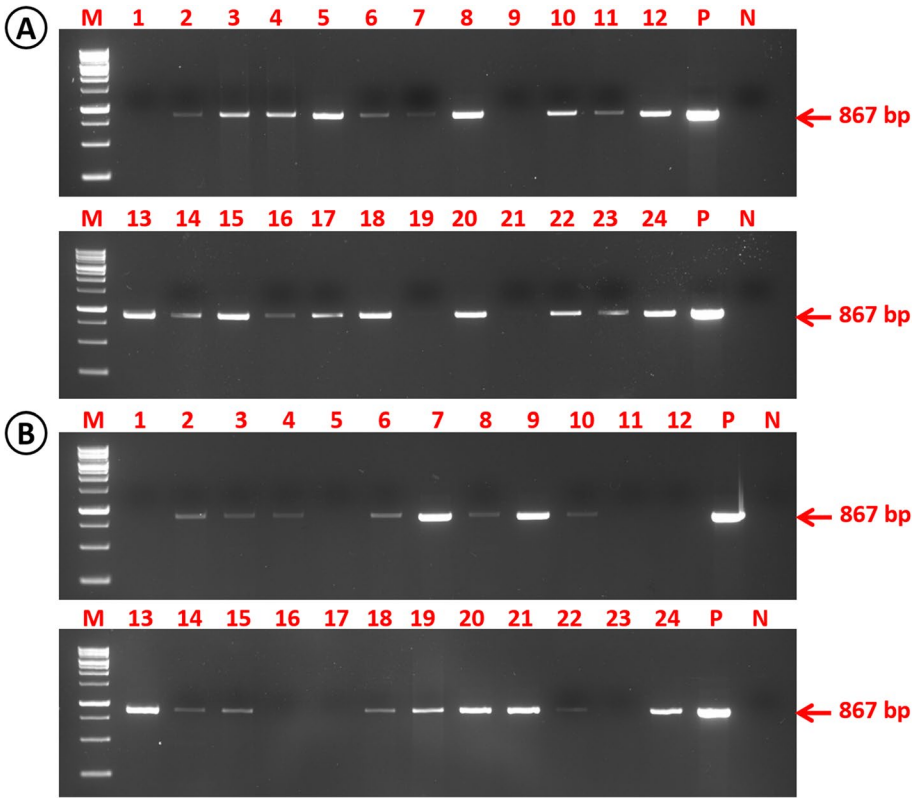

Fig. 2 Detection of TuMV infection in mustard seedlings via RT-PCR using CP-specific primers. **A** (lane 1–24) of Pusa Gold. **B** (lane 1–24) of Pusa Karishma. Lane M- 1 kb DNA ladder marker, lane P- positive control (plasmid), lane N- negative control

seedlings of Pusa Gold, BN-3, Pusa Karishma, Pusa Vijay, and PDZ-11 were sequenced and submitted in NCBI. The sequences obtained were found to share a sequence identity of more than 98 per cent with the nucleotide sequence of the CP gene of GRJCJ09 isolate of TuMV (JF314848). The nucleotide and protein sequences were also 100% identical to the TuMV genome assembled using HTS in this study.

Discussion

The seed transmission offers a mode of horizontal plant virus transmission and many potyviruses have been reported to be seed transmitted till date [4]. However in case of turnip mosaic potyvirus, earlier studies lack clarity on the effective seed transmission of TuMV in Arabidopsis, radish, and probably oilseed rape [26–28]. TuMV was found to be 4% seed transmitted in radish, but the plants grown from such infected seeds were observed to be disease-free [26]. Recently, it was claimed that accessions of *B. napus*, *B. juncea*, and *B. rapa* were susceptible to TuMV seed transmission, with up to 40% of seed embryos being contaminated [27]. However, the infectivity of the virus present in the embryos was never tested. The presence of a virus in the seed embryo does not guarantee that the seedlings will be infected. In contrast, its transmission from seed to seedlings was conclusively shown at levels up to 5% of seedlings of Arabidopsis [28]. High light intensity enhanced the TuMV seed transmission rates in Arabidopsis [29]. In this study, the seed transmissibility of TuMV, a highly infectious potyvirus, was hypothesized in mustard, an annual oilseed crop that is grown all around the world. The seed transmission tests using RT-PCR analysis of RNA isolated from immature seeds harvested from naturally TuMV-infected mustard plants followed by sequencing confirmed the seed-borne nature of TuMV. Also, the seedlings grown from mature seeds obtained from these plants under vector free protected conditions confirmed the seed transmissibility of TuMV in all three types of mustard species i.e. brown, black and yellow mustard for the first time. The sequence identity analysis confirmed it to be the TuMV Indian isolate that belonged to the World-B group [24].

Although embryonic infection is a need for the majority of seed transmission events, it does not always lead to seedling infection [4]. In actuality, there can be significant differences in the rates of seed infection and seed-to-seedling transmission. For instance, for zucchini yellow mosaic virus (ZYMV), the rate of seed infection was much greater (21.9%) than the percentage of seed-to-seedling transmission (1.8%) [30]. Similar results were found with lettuce mosaic virus (LMV) [31]. However, in this study, contrasting trends were observed in seed transmission rates of TuMV, which were observed to be as high as 84% and 72% in Pusa Gold and Pusa Karishma, respectively. The high rate of seed transmission in the current study may be due to the repeated use of seeds collected from the previously sown fields, allowing the perpetuation of the virus inoculum in the same field along with vector transmission. The high seed transmission rates demonstrated here could also be due to the limited number of plants used for testing, but these numbers could vary when more plants are subjected to virus detection. Earlier, 40% infection of seed embryos with TuMV I2 strain has been demonstrated, but the results of seed-to-seedling transmission were not clearly demonstrated [27]. Even though we found the virus in immature seeds and determined its presence to be restricted to embryos and cotyledon, there is a need to compare the rate of virus infection in immature and mature seeds.

Some potyviruses have been demonstrated to affect the seed germination rate in host plants to as low as 22.5%, such as ZYMV [30]. However, germination studies of two genotypes of mustard infected with TuMV exhibited germination up to 90%, and further studies need to be done taking seed germination count from healthy plants into consideration. Generally, seedlings grown from infected seeds may or may not produce symptomatic first-generation plants [32, 33]. In our study, leaf puckering, distortion and stunting symptoms were observed in mustard plants vertically infected with TuMV in 20–30 days old plants, depending upon the variety. The appearance of characteristic symptoms of stunting and puckering in the plants grown in controlled conditions confirms the Koch postulates for TuMV to be established as the causative of the same disease and being seed borne. The re-testing of the immature seeds collected from NPF-grown plants and confirmation of the presence of TuMV, further confirmed its true seed transmissible nature. The necessity to adopt strict phytosanitary measures for these viral seed infections will become increasingly crucial given that seed transmission plays a pivotal role in the epidemiology of viral diseases since it acts as a method of dispersal via seeds and establishes a source of inoculum for vector dispersal [34]. It is vital to comprehend how seed transmission rates translate into epidemics because viral infections have the capability to start epidemics since they're the most prevalent emerging pathogens in plants.

Conclusion

This study demonstrated the true seed-borne nature of TuMV through its presence in the embryo. In addition, the cotyledon was found to be associated with TuMV. The infectivity was also transmitted to the next-generation plants, as indicated by the grow-out test and symptom expression in the first-generation plants

raised from infected seeds. This study is the first report of TuMV seed infection of mustard species and vertical transmission to seedlings and to mature plants. This result is significant because mustard is an important oilseed and spice crop cultivated worldwide and is a symptomatic host of TuMV, causing varied losses all over the world. The seed transmissible nature may pose a threat to mustard cultivation worldwide.

Abbreviations

TuMV	turnip mosaic virus
CMV	cucumber mosaic virus
RT-PCR	reverse transcription polymerase chain reaction
CP	Coat-protein
ICAR-IARI	Indian Council of Agricultural Research-Indian Agricultural Research Institute
HTS	High-throughput sequencing
NPF	National Phytotron Facility
cDNA	complementary DNA
ZYMV	zucchini yellow mosaic virus
LMV	lettuce mosaic virus

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10866-2.

Supplementary Material 1: Fig. S1. a) Seeds sown in sieved autoclaved soil filled pots kept in growth chamber in National Phytotron Facility. b) 1st generation plants grown from seeds of infected plants left from the stock of tested plants for seedling transmission.

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Not applicable.

Compliance with ethical standard
This article follows the experimental guidelines of the country.

Authors' contributions
Pankhuri Singhal: Conceptualization, Methodology, Validation, Formal analysis, Writing - original draft. Damini Diksha: Methodology, Validation, Virendra Kumar Baranwal: Conceptualization, Methodology, Formal analysis, Writing - review & editing, Resources, Supervision. Naveen Singh: Resources, Supervision. Amalendu Ghosh: Methodology, Validation, Formal analysis.

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Data availability
The TuMV coat protein nucleotide sequences have been deposited in NCBI. Immature Seed sample JC-21 - OP132406Seedling Pusa Gold - OP132407Seedling BN-3 - OP132408Seedling Pusa Karishma - OP132409Seedling Pusa Vijay - OP132410Seedling PDZ11 - OP132411.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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