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ORIGINAL ARTICLE

Occurrence of *Banana bract mosaic virus* on *Musa ornata* Roxb based hybrids in India

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Abstract *Musa ornata*, wild species of banana is being used as a cut flower, potted plants and for landscape gardening etc., They are also being utilized in banana hybridization programmes for introgressing pest and disease tolerant traits into banana cultivars in addition to the development of inter specific ornamental banana hybrids. Symptoms of *banana bract mosaic virus* (BBrMV) was observed in the bracts of interspecific *M. ornata* based hybrid developed using another wild species i.e., *Musa rubra* Kurz at ICAR-National Research Centre for Banana (NRCB), Tiruchirapalli. Presence of the virus in the bracts, leaves and roots of symptomatic plants was confirmed through triple antibody sandwich enzyme linked immunosorbent assay with BBrMV monoclonal and polyclonal antibodies. BBrMV HC-Pro (1370 bp), CP (900 bp) and VPg (570 bp) genes were amplified from the infected bracts using reverse transcriptase polymerase chain reaction with BBrMV respective gene primers. The amplicons of these three genes were cloned and sequenced. Blastn analysis revealed that HC-Pro, VPg and CP gene sequences has 97.67%, 97.72% and 99.67% similarity with the respective gene sequences of BBrMV infecting banana. Phylogenetic analysis clustered the test isolate with other BBrMV isolates of banana and other hosts based on CP and HC-Pro and VPg gene sequences. The virus is transmitted through *Pentalonia nigronervosa* and the transmitted plants expressed symptoms under glass house conditions. To the best of our knowledge, this is the first report of BBrMV on ornamental *M. ornata* hybrid in India and its transmission occurs through *Pentalonia nigronervosa*.

Keywords *Banana bract mosaic virus* · *Musa ornata* hybrid · *Pentalonia nigronervosa* · Coat protein · Helper component proteinase · Viral genome-linked protein

Introduction

Wild species of *Musa* distributed in India viz., *M. ornata*, *M. velutina*, *M. acuminata*, *M. sabuana*, *M. arunachalensis*, *M. cylindrica*, *M. balbisiana* var. *elavazhai*, *M. chunii*, *M. laterita*, *M. cheesmanii*, *M. flaviflora*, *M. mannii*, *M. nagensium*, *M. ochracea* and *M. thomsonii* are known for their ornamental value and are being used in home garden and nurseries [10–12, 19, 21, 22, 28]. Constant creation of novelty in ornamental plants is the prime demand of world ornamental market. Besides the usage of banana as food, utilization of their ornamental features in the production of novel ornamental bananas not only increases their value but can also be a best alternative to meet the demand of ornamental industry. Keeping this in view, wild species of banana are being used in hybridization programme worldwide with an aim to develop attractive ornamental bananas for landscaping [33]. *M. ornata* Roxb., a well-known ornamental banana species grows up to 2 m height, producing attractive flowers with pale pink or lilac bracts. Distribution of *M.*

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ornata is reported from India, Bangladesh, and Myanmar. It is exported as an ornamental plant throughout the world due to its aesthetic nature [9]. It is being used as a donor parent in hybridization programme due to its ornamental feature and tolerance to many pests and diseases [6]. *M. rubra* Wall. ex Kurz. distributed from North-East India, Myanmar to Western Thailand. This species grows as undergrowth and on slopes of the forest margin and produces male flower with attractive red coloured bracts. To produce ornamental bananas with attractive novel ornamental characteristics, over 100 interspecific hybrids were developed at ICAR-NRCB, Tiruchirapalli by crossing *M. ornata* and *M. rubra* [8]. The hybrid plants raised from this cross produce male flowers with attractive colour, persistent non-revolute bracts with long and erect inflorescence (rachis). The development of these characters in the male flower of *M. ornata* hybrids increases their ornamental value and usage as cut flowers. Banana bract mosaic disease incited by the banana bract mosaic virus (BBrMV) is a serious disease and cause severe economic loss in banana. BBrMV develops characteristic spindle-shaped, purplish streaks on bracts, pseudo stems, midribs, peduncles, and even fruits [14]. The occurrence of bract mosaic disease caused by BBrMV was first noticed in the *M. ornata* hybrid grown in a research farm, ICAR-NRCB, Tiruchirapalli during 2020. The incidence of the disease in *M. ornata* hybrid is ca.7% was noticed. BBrMV belongs to the genus *Potyvirus* of the family *Potyviridae*. The virus is flexuous and filamentous particles measuring 750 × 11 nm with a single-stranded positive-sense RNA genome consists of 9711 nucleotides [5]. Other than the main host, BBrMV also infects abaca (*Musa textilis*), small cardamom (*Elettaria cardamomum*), and flowering ginger *(Alpinia purpurata*) [25, 27, 34]. To the best of our knowledge, the occurrence of this disease in ornamental banana hybrid is the first time noticed in India. Hence, an investigation was carried out to explore the occurrence of BBrMV in *M. ornata* hybrids, characterize the BBrMV isolate at a molecular level and study the transmission of the virus by aphids under glasshouse conditions.

Materials and methods

Collection of samples

A disease characterized by typical spindle-shaped mild streaks on flower bracts of *M. ornata* hybrid (MOH) was observed at Indian Council of Agricultural Research-National Research Centre for Banana (ICAR-NRCB) Research Farm, Tiruchirapalli during 2020 (Fig. 1a). Various plant parts viz., bracts, leaves, pseudo stem tissue, and roots from the BBrMV infected as well as healthy *M. ornata* banana hybrid plants were collected and kept immediately in ice. The samples were taken to the laboratory and then stored at − 80 °C. The reverse transcription-polymerase chain reaction (RT-PCR) tested BBrMV-infected (positive control) and BBrMV free (negative control) banana plants of cv. Nendran maintained in an insect-proof greenhouse, ICAR-NRCB, Tiruchirappalli, India were used.

Fig. 1 **a** Symptoms of banana bract mosaic disease in ornamental banana *Musa ornata* Hybrid; **b** Aphid transmission of BBrMV. A banana plant of *M. ornata* hybrid grown in pot culture expressing typical BBrMV symptoms through the transmission of BBrMV by *Pentalonia nigronervosa*

Diagnosis of BBrMV using triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA)

The samples collected from healthy and BBrMV infected *M. ornata* hybrid were tested by TAS-ELISA using polyclonal and monoclonal antiserum produced at the Molecular Virology lab, ICAR- NRCB for detection of BBrMV. Briefly, the wells of the polystyrene plate were first coated with BBrMV polyclonal antiserum and kept for incubation at 4 °C overnight. The wells were then washed with phosphate buffer saline (PBS) coated with the antigen extracted from the samples using antigen extraction buffer and the plate was incubated at 37 °C for 2 h. BBrMV monoclonal antiserum produced in mouse was added (1:1000 dilution) to the wells after washing wells with PBS and the plate was kept at 37 °C for 1 h. The wells were then washed with PBS, coated with anti-mouse IgG conjugated with alkaline phosphatase (1:2000 dilution), and incubated at 37 °C for 1 h. In the last step, wells were washed with PBS and the plate was incubated at 37 °C for 1–2 h after adding the substrate p-nitrophenyl phosphate (1.0 mg/ml; Sigma-Aldrich, St. Louis, MO). After incubation with PNPP at 37 °C for 2 h in the dark, absorbance at 405 nm was measured using a Synergy H1 microplate reader (Biotek, USA). A reaction was considered positive only if the average optical density (OD) of the sample was two times higher than the mean of the negative control. The positive and negative controls were included in each test.

RNA extraction, PCR amplification, cloning and sequencing

Total RNA was extracted from 100 mg of the symptomatic flower bracts using the Spectrum™ Plant Total RNA Kit according to the manufacturer's instructions (Sigma-Aldrich, USA) and complementary DNA (cDNA) strand was synthesized with an oligo(dT) primer as described by Balasubramanian and Selvarajan [2]. The Helper component-proteinase (HC-Pro), viral genome-linked protein (VPg), and coat protein (CP) genes of the BBrMV were amplified from the template cDNA through polymerase chain reaction (PCR) with gene-specific primer pairs. These genes were amplified using a thermal cycler (Applied Biosystems Veriti 96-Well Thermal Cycler, Thermo Scientific, USA) with the thermal cycling conditions as described earlier [1, 3, 4]. Amplicons were analyzed by electrophoresis in 1.5% agarose gels in TBE buffer, visualized by staining with ethidium bromide, and recorded with Gelstan Chemiluminescence and Fluorescence Imaging System (Medicare, Chennai). The sizes of the PCR products were determined by comparison with a standard 1 kb molecular marker (Thermo Scientific™ GeneRuler™, USA). The amplified RT-PCR products were purified using GeneJET PCR Purification kit (Thermo Scientific, USA) in accordance with the manufacturer's protocol, cloned into pGEMT-T easy vector (Promega, USA) and selected clones were sequenced in both directions with forward and reverse primers (SP6 and T7) using an automatic sequencer (Eurofins Genomic India Pvt Ltd., Bangalore).

Sequence and phylogenetic analysis

Nucleotide sequence comparisons of BBrMV VPg, CP and HC-Pro genes were detected using the Basic Local Alignment Search Tool (BLAST) against a database (https://www.ncbi.nlm.nih.gov/). All three gene sequences of BBrMV infecting *M. ornata* hybrid were deposited in the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov/). Alignments of sequences of BBrMV VPg, CP and HC-Pro genes at nucleotide (nt) and amino acid (aa) levels with respective gene sequences of BBrMV infecting different crops retrieved from the NCBI database were done using CLUSTALW [31]. The pairwise nt and aa sequence identity scores were represented as color-coded blocks using SDT v.1 software [16]. The evolutionary distance and phylogenetic analyses were conducted using a Maximum-likelihood phylogenetic tree construct in the MEGA 7.0 software.

Transmission of BBrMV by aphids

Transmission of BBrMV by the banana black aphids, *P. nigronervosa* was conducted in the glass house conditions as per the method described by Munez [17]. Briefly, non-viruliferous banana black aphids were placed on detached bracts of *M. ornata* hybrid infected with BBrMV and allowed to feed for 5 min acquisition access period (AAP). Those 50 or more aphids were transferred to ten healthy banana seedlings confirmed by RT-PCR (~ 10 aphids in each plant) of the same hybrids and allowed to feed for 30 min inoculation access period (IAP), and then the aphids were killed with an insecticidal spray. The plants were maintained in an insect-proof cage at 25–30 °C for 30 days in the glass house and the plants were inspected daily for symptom development. Simultaneously we performed transmission of BBrMV from *M. ornata* hybrid to BBrMV free healthy Grand Naine tissue culture banana plants.

Results and discussion

ELISA has been successfully employed by researchers in various years to detect BBrMV in infected banana tissue [13, 24, 29, 30]. In the present study, the presence of BBrMV in various parts of the BBrMV infected *M. ornata* hybrid plant was detected using TAS-ELISA. The development of a clearly visible yellow colour in the wells of the ELISA plate confirmed the presence of the BBrMV in the samples. However, colour development was not observed in the healthy samples indicating the absence of virus in healthy samples as expected. The optical density (OD) values of BBrMV infected main plant parts of bract, young leaf and pseudo stem tissue samples was between 0.519 and 1.217 whereas the OD values of the samples of infected sucker was between 0.588 and 1.644. The values for bract of the infected mother plant and, roots and the young leaf of infected sucker were significantly higher than the negative control OD values. Maximum OD was observed in roots of infected daughter sucker (1.644) followed by a bract (1.217) of the infected mother plant, young leaf (0.927) of infected daughter sucker and the lowest value was observed in the pseudostem tissues of infected mother plant. OD values of 0.124, 0.137, and 0.122 were obtained from bract, young leaf, and pseudostem tissue of healthy samples confirmed by RT-PCR respectively, which are considered as negative for the virus as the OD values do not exceed two times more than the negative control. The presence of the virus was detected in bracts of the infected mother plant and, the young leaf and roots of the infected daughter sucker, but viral titer varied among the tissues (Table 1).

The HC-Pro, VPg, and CP are the multifunctional proteins of potyvirus. These proteins were found to be involved in the infection cycle of the virus [7, 18, 20, 26, 32]. Characterization of these genes and their sequence comparison was used to identify and find the diversity and phylogenetic relationship of BBrMV isolates [1, 3, 4]. In

Table 1 Absorbance values for different plant parts of MOH were assayed for Banana bract mosaic virus using triple antibody sandwich ELISA (TAS-ELISA)

Type of sample	Absorbance value at 405 nm*	
	Healthy	BBrMV Infected
Bract of the mother plant	0.124	1.217
Young leaf of the mother plant	0.137	0.734
Pseudostem of the mother plant	0.122	0.519
Young leaf of daughter sucker	0.163	0.927
Pseudostem of daughter sucker	0.148	0.588
Roots of daughter sucker	0.152	1.644
Positive control	–	3.340
Negative control	–	0.132

*Average of three replications

this study, we have amplified HC-Pro, CP, and VPg genes of BBrMV infecting *M. ornata* hybrid and analysed its divergence with other host BBrMV isolates at the genetic level using these gene sequences. RT-PCR gave expected amplicons of 1370 bp (HC-Pro), 570 bp (VPg), and 900 bp (CP) of BBrMV from the symptomatic bracts of MOH (Supplementary Fig. 1). These amplicons were cloned and sequenced. The size of HC-Pro, VPg, and CP genes was similar to the previously published sequences [18–20]. The VPg and CP gene sequences had 97.72% and 99.67% similarity with the respective gene sequences (KT852552 and KY753432) of BBrMV infecting banana whereas HC-Pro gene sequences had 97.67% similarity with HC-Pro gene sequences (MG758140) of BBrMV infecting cardamom. These results confirm the presence of BBrMV in the symptomatic *M. ornata* hybrid. The sequences of the BBrMV infecting ornamental *M. ornata* hybrid were submitted in GenBank, NCBI and provided with accession numbers, OK315655, OK315656, and OK315657 for CP, HC-Pro, and VPg genes respectively.

Pairwise sequence analysis of the HC-Pro gene sequence of MOH isolate reported in the present study shared 94–98% at the nucleotide (nt) level and 93–98% identity at the deduced amino acid (aa) level. MOH isolate shared maximum identity (98%) with AP1, KAR2, TN25, TN31, and AS1 isolates and minimum identity (94%) with the Philippines isolate at nt and except TN32, all isolates shared 98% similarity at aa level (Supplementary Fig. 2). Pairwise identity of VPg gene of MOH isolate exhibited a range of nt and aa identity of 95–98% and 98–99%, respectively. This isolate shared the least sequence identity of 95% with Philippines banana isolate, *Alpinia purpurata,* and *Musa textiltis* and the remaining isolates shared 98% at nt level. Similarly, deduced aa sequence of the isolate under this study shared a maximum identity of 99% with BBrMV isolates of banana from the Philippines and *M. textiltis* and 98% with other isolates

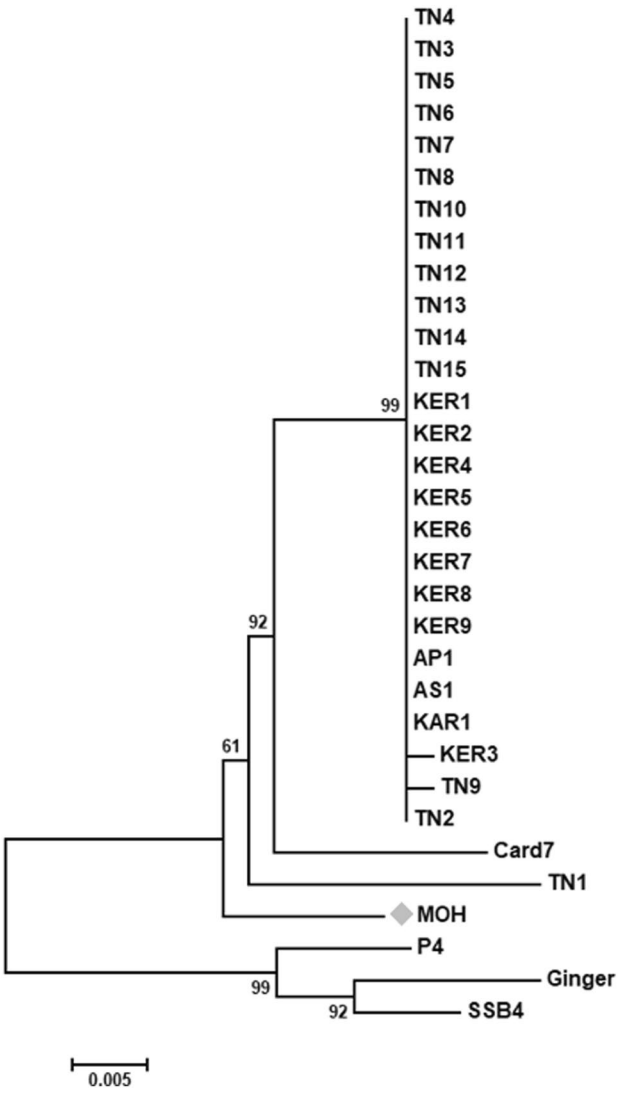

Fig. 2 Phylogenetic analysis of nucleotide sequences of the BBrMV viral genome linked protein (VPg) with corresponding regions of other BBrMV isolates by using the Maximum Likelihood method based on the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap scores above 50% (1000 replicates) are placed at the tree nodes. For the details of isolates, refer to Supplementary Table 1

available in the GenBank (Supplementary Fig. 3). Furthermore, pairwise CP gene sequence alignment revealed the MOH isolate under this study shared the sequence identity of 80–100% and 84–99% at nt and aa levels. The MOH isolate reported the highest identity of 99–100% and the lowest is 80–84% with isolates of TN4 and TN14 at aa and nt levels (Supplementary Fig. 4).

A phylogenetic tree was constructed by the maximum likelihood method based on HC-Pro nt sequence alignment of all 27 available BBrMV isolates revealed closeness of BBrMV-MOH isolate with TN7 isolate. The isolates P4 and *A. purpurata* were grouped in one cluster (Group I) while

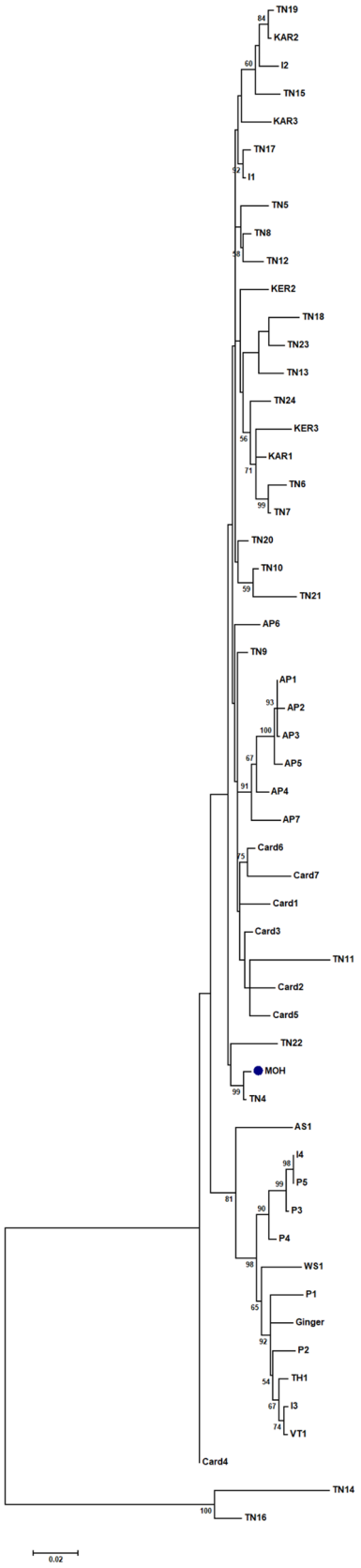

Fig. 3 Phylogenetic analysis of nucleotide sequences of the BBrMV coat protein (CP) with corresponding regions of other BBrMV isolates by using the Maximum Likelihood method based on the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap scores above 50% (1000 replicates) are placed at the tree nodes. For the details of isolates, refer to Supplementary Table 1

TN29 and TN32 were grouped in one cluster (Group II) and the remaining other isolates formed another cluster (Group III) (Supplementary Fig. 5). Phylogenetic analysis of the VPg gene nt sequences of all 32 BBrMV isolates revealed that P4, *M. textiles* (SSB4), and *A. purpurata* were grouped in one cluster whereas BBrMV MOH was grouped with TN1 and Card7 isolate and remaining all other India banana isolates were grouped closely into one cluster (Fig. 2). Nucleotide sequences of all 54 BBrMV isolates were used for phylogenetic analysis with the MOH isolate for the CP gene. Indian isolates from either banana or cardamom exhibited great divergence, while all the isolates from Southeast Asia, Ecuador, Samoa, and Hawaii were grouped closely into the same close clade (Fig. 3). Phylogenetic analysis shows that BBrMV infecting *M. ornata* hybrid grouped in different groups along with other isolates of BBrMV based on the differences in nucleotide sequences irrespective of their host and geographic origin. The genetic diversity among the BBrMV isolates had no relationship with their geographical origin as well as the host from which they were isolated [1, 3] and it is not known how the BBrMV from banana has spread to small cardamom and flowering ginger. As all these species belongs to Zingiberacae, this virus would have been transmitted through banana aphids which can also colonize these hosts. These results also indicate that BBrMV infecting ornamental banana hybrids may be able to infect other banana cultivars and also other host species through a common banana aphid vector. However, a cross infectivity test must be done to confirm the infection of BBrMV of *M. ornata* hybrid on other hosts and vice versa.

BBrMV was reported to be transmitted non-persistently through several aphid species such as *P. nigronervosa*, *Rhopalosiphum maidis*, *Aphis gossypii*, and *Aphis craccivora* [15, 17]. Results of the present study revealed that *P. nigronervosa* could transmit the virus in a non-persistent manner with 5 min AAP and 30 min IAP as evidenced by the production of characteristic light green coloured spindle-shaped streaks on the leaves of 35% of total *M. ornata* hybrid plants (Fig. 1b). The rate of transmission of BBrMV from *M. ornata* hybrid to banana cultivar Grand Naine plants was 40%. Expression of the symptoms was observed on the plants after 30 days from inoculation and the presence of the virus in the symptomatic plants was confirmed by using TAS-ELISA (Data not shown).

To our knowledge, this is the first report of BBrMV on ornamental *M. ornata* hybrids in India and its transmission

through *P. nigronervosa*. The aesthetic look of the ornamental banana is based on its attractive inflorescence and its shining nature. The infection of the BBrMV in plants results in streaks on flower bracts leading to necrosis. The infected flower buds wither and fall of bracts occur early before their maturity from the flower and thereby reduction in their ornamental values. At present, the *M. ornata* hybrid is propagated only through suckers. The suckers collected from the infected mother plants may act as a source of inoculum for the virus spread and infection. TAS-ELISA results of the present study also show that the load of BBrMV is more in the roots and young leaves of infected daughter suckers than in the infected mother plant. Hence, necessary steps must be taken to control the spread of this virus into other parts of India and to other countries in which BBrMV is a quarantine pathogen. It is very much necessary to devise suitable disease management practices before it turns out to be a potential threat to the ornamental banana industry. *M. ornata* is utilized in hybridization programmes to develop pest and disease-resistant banana hybrids as it is having pest and disease tolerance traits, possesses male and female fertility, and produces viable seeds upon pollination with other *Musa* species. In this study infection of *M. ornata* hybrid with BBrMV and its presence in most parts of the plant was detected. In addition, the seed transmission nature of this virus is demonstrated in banana synthetic diploid, H-201 [24]. However, the seed transmission nature of BBrMV with *M. ornata* must be studied thoroughly before its successful utilization in the hybridization programme for varietal development.

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Data availability Data will be available on request.

Declarations

Conflict of interest All authors declared no conflict of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval Not applicable.

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