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Identification and Validation of Quantitative Trait Loci of Mungbean Yellow Mosaic Virus Disease Resistance in Blackgram [*Vigna mungo* (L). Hepper]

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ABSTRACT

Background: Blackgram [*Vigna mungo* (L.) Hepper] is an important food legume crop of India. Mungbean yellow mosaic virus (MYMV) disease is the major problem in blackgram. The disease can reduce seed yield upto 100% or even kill a plant infected at an early vegetative stage. The most effective way to prevent the occurrence of this disease is to develop genetically resistant cultivars of blackgram. However, a major difficulty in breeding MYMV disease resistant in blackgram is field screening for the virus disease. Hence identification of QTL followed by Marker-assisted selection (MAS) is highly useful for genetic improvement of crops. With this background, a study was made for identification as well as validation of quantitative trait loci (QTL) for MYMV disease resistance in blackgram.

Methods: A total of 112 $F_{2:3}$ lines were evaluated for MYMV disease resistance along with parents *viz*., MDU 1 (MYMV disease susceptible) and Mash 1008 (MYMV disease resistant) at the National Pulses Research Centre, Tamil Nadu Agricultural University, Vamban, Tamil Nadu during July-September 2018 under Augmented Design in the field. Each line was sown in one row of 3 m in length with a spacing of 30 cm as between row and 10 cm as within row. Susceptible genotypes CO 5 and MDU 1 were sown as disease spreader rows after every eight rows and also around the plots. The MYMV disease score was recorded on 60 DAS, by using phenotype rating scale from 1 (resistant) to 9 (highly susceptible), as suggested by Singh *et al.* (1995). The mean of each progeny was calculated and used as phenotypic data. A total of 525 SSR primers were used to test polymorphism between parents MDU 1 and Mash 1008. Genotyping was carried out for 112 $F_{2:3}$ RILs of the cross MDU 1 x Mash 1008 with 35 polymorphic SSR markers. Linkage and QTL analyses were performed using QTL IciMapping (version 4.1.0.0) (Wang *et al.* 2016) and QGene 4.4.0 (Joehanes and Nelson 2008) respectively. Two mapping populations MDU 1 x Mash 114 and CO 5 x VBN 6 in $F_{2:3}$ and F_2 generations respectively were used in this study to validate the identified QTL regions.

Result: QTL study indicated the presence of two major QTLs for MYMV disease score in LG 2 and LG 10 at 60 DAS with 20.90 and 24.90% of phenotypic variation respectively. Validation of these QTLs in two other mapping population indicated that QTL on LG 10 was validated with high phenotypic variation of 45.40-46.00%. Hence it may conclude that *qmymv10_60* may be utilized for MAS/ MABC with assured improvement on MYMV disease resistance in blackgram.

Key words: Blackgram, Disease, MYMV, QTL, SSR.

INTRODUCTION

Blackgram [*Vigna mungo* (L.) Hepper] is an important food legume crop of India. It is an important short duration crop and widely cultivated in India. It gives an excellent source of easily digestible good quality protein and ability to restore the fertility of soil through symbiotic nitrogen fixation. The seeds are highly nutritious with protein (24-26%), carbohydrates (60%), fat (1.5%), minerals, essential amino acids and vitamins. The nutritional value improves greatly, when wheat or rice is combined with blackgram because of the complementary relationship of the essential amino acids such as arginine, leucine, lysine, isoleucine, valine and phenylalanine *etc*. (Mehra *et al.,* 2016). In India, the cultivated area under blackgram is about 4.50 million hectares with the production of 2.83 million tonnes (AICRP on MULLaRP, 2018). Mungbean yellow mosaic virus (MYMV) disease is the major problem in blackgram. The disease is economically important, destructive, wide spread and inflicts heavy losses annually. The disease mainly occurs in South Asian countries India, Sri Lanka, Pakistan and Bangladesh (Malathi and John

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2009). The disease can reduce seed yield upto 100% or even kill a plant infected at an early vegetative stage (Naimuddin, 2001). Mungbean yellow mosaic virus, a member of family Geminiviridae is transmitted by the polyphagous insect vector, whitefly (*Bemisia tabaci*). It has a bipartite genome (DNA A and DNA B) (Mansoor *et al.,* 2003; Jeske, 2009). Begomo viruses include mungbean yellow mosaic virus (MYMV), mungbean yellow mosaic India virus (MYMIV), Dolichos yellow mosaic virus (*Do*YMV) and horsegram yellow mosaic virus (HgYMV), which are wide spread throughout South Asia (Haq *et al.,* 2011). Both MYMV and MYMIV cause yellow mosaic virus (YMD) on blackgram. MYMV is prevalent in southern India where Tamil Nadu is situated (Nair *et al.,* 2017). In blackgram, MYMV diseases cause irregular yellow green patches on older leaves and complete yellowing of young leaves of susceptible varieties. The characteristic yellow mosaic symptoms include two types, yellow mottle and necrotic mottle that can be distinguished visually (Nair and Nene, 1974). Moreover, the control of MYMV disease is often based on limiting the vector population by insecticides, which are ineffective under severe whitefly infestations. In highly susceptible plants, symptoms include shortening of internodes, severe stunting of plants with no yield or few flowers and deformed pods producing small, immature and shriveled seeds. The most effective way to prevent the occurrence of this disease is to develop genetically resistant cultivars of blackgram. Hence understanding the inheritance pattern and the nature of gene action involved in MYMV disease reaction is essential. There are some conflicting reports about the genetics of resistance to MYMV disease claiming both resistance and susceptibility as dominant. In blackgram, monogenic dominant nature of resistance was reported by Dahiya *et al.* (1977), Kaushal and Singh (1988) and Gupta *et al.* (2005) while it was reported to be digenic recessive by Singh (1980), Dwivedi and Singh (1985) and Verma and Singh (1986). Monogenic recessive control of yellow mosaic resistance was also reported by some authors (Pal *et al.,* 1991). Improved resistance to MYMV is now the major goal for breeding programs in several blackgram and greengram production countries. However, a major difficulty in breeding MYMV disease resistant in greengram or blackgram is field screening for the virus disease. Hence identification of QTL followed by Marker-assisted selection (MAS) is highly useful for genetic improvement of crops. MAS can accelerate the development of new cultivars by reducing the number of generations and increasing precision in phenotypic evaluations (Collard and Mackill 2008). So far, there are few reports on DNA markers associated with mungbean yellow mosaic virus disease resistance in blackgram. With this background, a study was made for identification as well as validation of quantitative trait loci (QTL) for MYMV disease resistance in blackgram.

MATERIALS AND METHODS

Plant material and development of mapping population

A total of 112 $F_{2:3}$ recombinant inbred lines (RILs) were developed from a cross between MDU 1 (female parent) and Mash 1008 (male parent). MDU 1 is a high yielding, popular blackgram variety grown in Tamil Nadu, but highly susceptible to MYMV disease. This variety was developed at Agriculture College and Research Institute, Tamil Nadu Agricultural University, Madurai and released in 2014 for commercial cultivation in October to November season of Tamil Nadu (MYMV disease free season). Mash 1008 is a variety released at Punjab Agricultural University, Ludhiana and is resistant to MYMV disease. Mash 1008 was used as a male parent and crossed with MDU 1 during July-September 2017 to produce F_1 seeds. An F_1 hybrid plant confirmed through SSR marker was selfed during October to November, 2017 to produce F_2 seeds. A total of 112 plants were selfed to obtain F_3 seeds and used to raise as $F_{2:3}$ recombinant inbred lines (RILs).

Phenotypic data

A total of 112 $F_{2:3}$ lines were evaluated for MYMV disease resistance along with parents at the National Pulses Research Center, Tamil Nadu Agricultural University, Vamban, Tamil Nadu (10.363505° N, 78.902283° W) during July-September 2018 under Augmented Design in the field. Each line was sown in one row of 3 m in length with a spacing of 30 cm as between row and 10 cm as within row. Susceptible genotypes CO 5 and MDU 1 were sown as disease spreader rows after every eight rows and also around the plots. No insecticide was sprayed in order to maintain the natural white fly populations. The MYMV disease score was recorded on 60 DAS, by using phenotype rating scale from 1 (resistant) to 9 (highly susceptible) (Table 1), as suggested by Singh *et al.* (1995). The mean of each progeny was calculated and used as phenotypic data.

DNA isolation, SSRs and PCR condition

Total genomic DNA of each parental and F_2 plants were extracted from fresh young leaf tissue using the CTAB method (Lodhi *et al.* 1994). The collected samples were ground in pestle and mortar with preheated (65°C) 500 µl of CTAB buffer. Extracted samples were taken into eppendorf tubes and incubated in the water bath for 30 min at 65°C. After incubation, 300 µl of phenol: chloroform: iso-amyl alcohol (25:24:1) was added into tubes and inverted twice to mix and kept in centrifuge for10 min at 10000 rpm. The supernatant was collected in the fresh tubes. The tubes were added with 500 µl of chloroform: iso-amyl alcohol (24:1) and inverted twice to mix. The tubes were centrifuged for 5 minutes at 10,000 rpm. The aqueous layer was transferred in to the new eppendorf tubes. An amount of 0.7 volume of isopropanol (stored at -20°C) was added to each sample and inverted once to mix and kept overnight at 4°C. The samples were centrifuged at 8000 rpm for 10 min on the next day. The supernatant was discarded from each sample and the pellets settled in the bottom were air dried for 30 min. A quantity of 50 µl of TE buffer was added into each sample and stored overnight at 4°C. RNAse (3 µl) was added into each sample and kept at 65°C for 30 min. The DNA quality and quantity were checked on 0.8% agarose gel and DNA concentration was normalized to10 ng/µl.

The polymerase chain reaction (PCR) mixtures, containing 2 µl of 10 ng template DNA, 1.0 µl of 10 X Taq buffer (M/s Genei, India) + $MgCl_2$ (1.5 mM), 1.0 µl of dNTPs (2 mM), 1.0 µl of forward and reverse SSR primers (0.5 µM), 0.3 µl of taq polymerase (3 IU) and 4.7 µl of sterile double distilled water. The DNA was amplified in a thermocycler (M/s Eppendorf, Germany, model AG 6325) under the following conditions: 94°C for 4 min followed by 30 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1 min, with a final extension step of 72°C for 20 min. The PCR products were separated on 3% agarose gel and photographed using GELSTAIN 4x advanced gel documentation unit (M/s Medicare, India).

Linkage map and QTL analysis

Linkage analysis was carried out using QTL IciMapping (version 4.1.0.0) (Wang *et al.* 2016). A minimum LOD threshold of 3.0 and maximum distance of 50 cM were used for construction of linkage groups. Map distance in centimorgan (cM) values was calculated using Kosambi mapping function (Kosambi 1944). QTL analysis was performed using QGene 4.4.0 (Joehanes and Nelson 2008) following composite interval mapping (CIM) and automatic cofactor selection. LOD threshold significance for each QTL was calculated with1000 runs of a permutation test at P = 0.05.

Validation of markers linked with MYMV disease resistance

Two mapping populations MDU 1 × Mash 114 and CO 5 × VBN6 in $F_{2:3}$ and F_2 generations respectively were used in this study to validate the identified QTL regions. The material was screened for the MYMV disease reaction in the field conditions at National Pulses Research Centre, Tamil Nadu Agricultural University, Vamban, Tamil Nadu during July - Sep 2018. Normal cultural practices were followed, except the insecticide application. CO 5 and MDU 1 which are highly susceptible cultivars to MYMV disease were sown as disease spreader rows in every ninth row and around the perimeter of plots to increase sufficient disease pressure. The disease scores were recorded on 60 DAS as described by Singh *et al.* (1995).

RESULTS AND DISCUSSION

Analysis of phenotypic variation

In the present study, mean and variability parameters were estimated for MYMV disease scores and presented in Table 2. Parameters, skewness and kurtosis help the breeder to understand the nature of distribution of individuals in the population. MYMV disease score at 60 DAS had non-significant skewness which indicates no skewness for this trait. MYMV disease score at 60 DAS had non-significant kurtosis. It indicates the mesokurtic nature of this trait. The results indicated the presence of normal distribution for the MYMV disease scores at 60 DAS (Fig 1).

Construction of linkage map

A total of 525 SSR primers were used to test polymorphism between parents MDU 1 and Mash 1008. Of these, 315 were from mungbean (Isemura *et al.,* 2012; Gwag *et al.,* 2006; Somta *et al.,* 2008; Seehalak *et al.,* 2009; Tangphatsornruang *et al.,* 2009) and 210 from adzuki bean (Wang *et al.* 2004; Chankaew *et al.,* 2014). Among 525 markers only 35 (14.1%) showed polymorphism between parents. Genotyping was carried out for 112 $F_{2:3}$ RILs of the cross MDU 1 × Mash 1008 with 35 polymorphic SSR markers. Linkage analysis was performed using QTL IciMapping (version 4.1.0.0) (Wang *et al.,* 2016). Linkage groups were established using a minimum LOD score of 3.0, ordering by RECORD, rippled by SARF criterion with a window size of 5. Nine linkage

Table 1: Disease scales for scoring of mungbean yellow mosaic virus (MYMV).

Grade	Description	Reaction
1	No visible symptoms on leaves	Free
2	Small yellow specks with restricted spread covering 0.1-5% leaf area	Highly resistant (HR)
3	Mottling of leaves covering 6-10% leaf area	Resistant (R)
4	Yellow mottling covering 11-15% leaf area	Moderately resistant (MR)
5	Yellow mottling and discolouration of 15-20% leaf area	Moderately susceptible MS)
6	Yellow coloration of 21-30% leaves and yellow pods	Susceptible (S)
7	Pronounced yellow mottling and discoloration of leaves and pods, reduction in leaf size and stunting of plants covering 30-50% 0f foliage	Susceptible (S)
8	Severe yellow discoloration of leaves covering 50-75% of foliage, stunting of plants and reduction in pod size	Highly susceptible (HS)
9	Severe yellowing of leaves covering above of foliage, stunting of plants and no pod formation	Highly susceptible (HS)

(Reference: Singh *et al.*, 1995).

Table 2: Mean and variability parameters for MYMV disease scores at 60 DAS in RIL populations of cross.

Population	Mean	Variance	SE	Skewness	Kurtosis	Minimum	Maximum
MDU 1 × Mash 1008	3.95	2.82	1.68	0.25^{ns}	-1.02^{ns}	1.10	7.70

SE-Standard error.

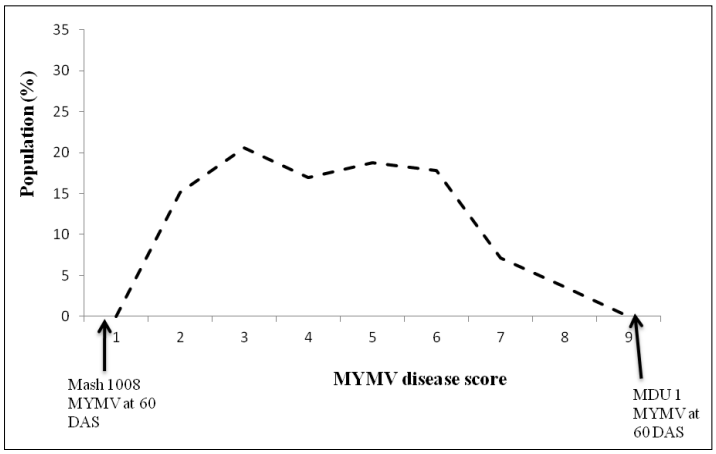

Fig 1: Frequency distribution for MYMV disease score at 60 DAS.

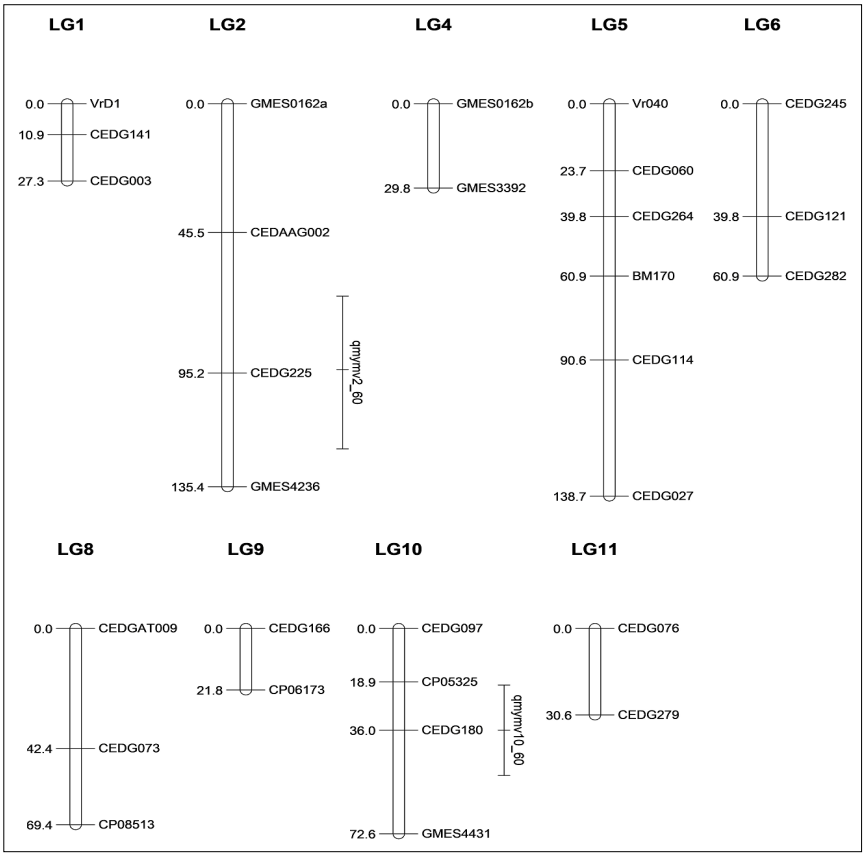

Fig 2: Linkage map of blackgram constructed with $F_{2:3}$ RIL population of MDU 1 × Mash 1008.

groups were established with 29 SSR markers (Fig 2). Remaining six markers were found as unlinked. The total length of the map was 586.08 cM.

QTLs of MYMV disease resistance

Composite interval mapping (CIM) was employed to locate QTL for MYMV disease resistance on the linkage map. The LOD threshold for MYMV disease resistance was determined by a permu-tation test with 1000 runs. Two QTLs for MYMV disease score at 60 DAS were located one each on LG2 and LG10 and designated as *qmymv2_60* and *qmymv10_60* (Fig 2 and Table 3) respectively. The *qmymv2_60* was flanked by CEDAAG002, CEDG225 and GMES4236, showing a LOD score of 5.71 and significant at *P* = 0.05. This QTL had an additive effect of 9.1 and explained 20.90% of variation for the MYMV disease score at 60 DAS. The *qmymv10_60* was flanked by cp05325, CEDG180 and GMES4431, showing LOD score of 6.98 and significant at *P* = 0.05. This QTL showed an ad-ditive effect of 9.0 and accounted for 24.90% of the vari-ation for the MYMV disease score at 60 DAS. In all cases, alleles from the resistant parent

Table 3: QTL mapping of $F_{2:3}$ RIL population of cross MDU 1 × Mash 1008 for MYMV.

QTLs	LG	Peak position (cM)	Marker interval	LOD	PVE	Additive effect	Dominance effect
qmymv2_60	2	94.0	CEDAAG002-CEDG225-GMES4236	5.71	20.90	9.10	-0.50
qmymv10_60	10	36.0	cp05325-CEDG180-GMES4431	6.98	24.90	9.00	4.70

LG- Linkage group; LOD- Logarithm of the odds ratio; PVE- Per cent of variation explained.

Table 4: Validation of MYMV QTLs in two crosses.

QTLs	LG	Peak position (cM)	Marker interval	LOD	PVE	Additive effect	Dominance effect
MDU 1 × Mash 114 ($F_{2:3}$)							
qmymv10_60	10	6.0	cp05325- CEDG198	5.12	45.40	9.67	-2.91
CO 5 × VBN 6 (F_2)							
qmymv10_60	10	12.0	CEDG198-CEDG180- CEDG097	9.16	46.00	2.63	-1.61

LG- Linkage group; LOD- Logarithm of the odds ratio; PVE- Per cent of variation explained.

Mash 1008 contributed towards the reduction in disease score, *i.e.* increasing resistance.

Validation of markers linked with MYMV disease resistance

A total of 80 SSR primers of LG 2 and LG 10 were used to test polymorphism between parents MDU 1, CO 5, Mash 114 and VBN 6. Of these, 55 and 25 primers were associated with LG 2 and LG 10 respectively. Among 55 markers of LG 2 only two showed polymorphism between parents in both crosses. Three and four primer showed polymorphism in LG 10 for the crosses MDU 1 × Mash 114 and CO 5 × VBN6 respectively. Genotyping was carried out for 39 $F_{2:3}$RILs and 68 F_2RILs of the crosses MDU 1 × Mash 114 and CO 5 × VBN6 respectively.

The LOD threshold for MYMV disease resistance was determined by a permutation test with 1000 runs. One QTL *qmymv10_60 was* detected by CIM for MYMV disease score at 60 DAS in both populations (Table 4). The *qmymv10_60* was flanked by cp05325 and CEDG198 with LOD score of 5.12 and significant at *P* = 0.05. It had an additive effect of 9.67 and explained 45.40% of variation in the MYMV disease score at 60 DAS in the cross MDU 1 × Mash 114. In CO 5 × VBN 6 cross combination *qmymv10_60* was flanked by CEDG198, CEDG180 and CEDG097 at LOD scores 9.16 on LG10. It had an additive effect of 2.63 and explained 46.00% of variation for the MYMV disease score at 60 DAS.

Kang *et al.* (2005) reported that more than 80% of viral resistance in plants is controlled by single gene. In the present study, composite interval mapping (CIM) on $F_{2:3}$ RILs populations showed two major QTLs *qmymv2_60* and *qmymv10_60* for MYMV disease score at 60 DAS. These two QTLs *on LG2 and LG 10* explained more than 20 per cent of variation on MYMV disease score at 60 DAS. Hence these QTLs can be considered as highly robust and can be used in marker-assisted selection programme for MYMV disease resistance. In blackgram, resistance gene analog markers YR4 and CYR1 were reported to be linked with resistance to MYMIV (Maiti *et al.,* 2011). Marker CYR1 was also asso-ciated with resistance in mungbean. CYR1 is proposed as part of the candidate disease resistance (R) gene (Maiti *et al.,* 2011). However, markers YR4 and CYR1 were found as monomorphic between the parents MDU 1 and Mash 1008. Gupta *et al.* (2013) reported that the SSR marker CEDG180 was associated with a major gene control-ling MYMV disease resistance in blackgram and amplified an allele of 136 and 163 bp in resistant and susceptible parents respectively. In the present study, *qmymv10_60* has the marker *CEDG180.* But this marker amplified an allele of 136 and 163 bp for susceptible and resistant parents respectively. This type of deviation might be due to the influence of the third gene which may have inhibitory gene action.

In this study, identified QTLs were validated with two other mapping populations MDU 1 × Mash 114 and CO 5 × VBN 6 in $F_{2:3}$ and F_2 respectively. However, to validate the identified QTLs, SSR markers reported on LG 2 and LG 10 of *Vigna* group Azudiki bean (Han *et al.,* 2005), Greengram (Isemura *et al.,* 2012) and Blackgram (Chaitieng *et al.,* 2006) were screened for polymorphism in the parental lines of MDU 1 × Mash 114 and CO 5 × VBN 6 crosses. In the present study, composite interval mapping (CIM) detected one QTL *qmymv10_60* for MYMV disease score at 60 DAS in both populations. The validation study revealed that the *qmymv10_60* alone validated in both mapping population and explained very high phenotypic variation of 45.40-46.00%. Hence it may conclude that *qmymv10_60* may be utilized for marker assisted selection/ marker assisted backcross in other mapping population with assured improvement on MYMV disease resistance lines in blackgram.

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Authors' contribution

K. Vadivel performed the field experiments, measurements, data analysis and K. Vadivel and S. Ragul drafted the

manuscript, N. Manivannan and A. Mahalingam supervised the work, worked on the manuscript and aided in interpreting the results, N. Manivannan, A. Mahalingam, V.K. Satya and C. Vanniarajan were involved in planning. All authors provided critical feedback on research, analysis and manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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