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Full Length Research Paper

Assessment of somaclonal variation in sugarcane

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A study was conducted from 2006 to 2008 to assess the variability arising from callus regeneration and its vegetative transmission in a subtropical variety of sugarcane, CoJ 64. Qualitative and quantitative characters were evaluated in the field at maturity in each of the 3 years at the same location. The frequencies of variants were compared to those found in the original variety. The tissue culture process resulted in increased morphological variability among the produced somaclones. Their variability was manifested according to the data for stalk diameter, length, internodes, millable canes, leaf length, width and sucrose characters. The variation among the somaclones was confirmed in the three consecutive years.

Key words: Callus regeneration, vegetative transmission, sugarcane.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is one of the economically important crops widely cultivated in the tropics to subtropics and annually provides around 60 to 70% of the world's sugar (Shah et al., 2009). Unfortunately, the production of this crop retained by several biotic and abiotic stresses such as bacterial and fungal diseases, drought, salinity, freezing etc. The improvement of sugarcane plant resistance to these stresses is of great importance. Genetic potential of a variety plays an important role in determining the stress resistance, yield and quality of it.

Genetic variability is the key factor in any breeding method. The genetic variability created through conventional breeding techniques is slow and depend on recombination (Mascarenhas, 1991). In conventional breeding method, development of elite sugarcane cultivars with high sugar yield and disease resistance are often defeated by tight linkage between cane quality, cane yield and disease resistance etc. The developments in plant tissue culture have opened up new possibilities in creating genetic variability. The use of tissue culture for creation of somaclonal variation can be used to increase the speed of efficiency of the breeding process to improve the accessibility of existing germplasm of sugarcane and create new variation for crop improvement.

It has been recognized that all plants regenerated from tissue culture are not exact replicas of a parental form and exhibit variability in agronomic traits. This genetic alteration termed somaclonal variation (Larkin and Scowcroft, 1981), which is being exploited to shorten the time needed to produce new breeding lines with desirable traits. In sugarcane somaclones have been identified with important traits such as disease resistance (Heinz et al., 1977; Krishnamurthi and Tlaskal, 1974; Ramos et al., 1996; Sreenivasan et al., 1987), plant morphology (Heinz and Mee, 1969; Nagai et al., 1986) and chromosome number (Sreenivasan and Jalaja, 1982; Sreenivasan and Sreenivasan, 1984a, b).

Sreenivasan and Sreenivasan (1984a, b) reported that the variation noticed in economically important characters such as tillering and sucrose percent will also facilitate selection and utilization of the best genotypes for further hybridization.

Sugarcane variety, CoJ 64, developed through sexual breeding method, is a high yielding and high sugared subtropical variety. This variety is susceptible to the

Abbreviations: NMC, Number of millable canes; **RAPD,** random amplified polymorphic DNA; **PCR,** polymerase chain reaction.

major sugarcane disease, red rot. Apart from this draw back this variety is cultivating in many parts of sub tropical region in the country. Due to its continuous cultivation and usage in the breeding programmes, this variety has faced deterioration and its performance becomes poorer in the fields. The application of tissue culture techniques will, therefore, be influenced by the amount of variability or uniformity that can be expected from plants regenerated from cultured tissues. The variety, CoJ 64, has not been so far exploited through *in vitro* methods for its better performance and crop improvement.

The present study was therefore undertaken to evaluate the somaclonal variation in somatic embryo derived plants of sugarcane var. CoJ 64 through morphological, cytological and molecular methods.

MATERIALS AND METHODS

Plant materials

The material used for the study was a commercial sugarcane variety, CoJ 64, which is a subtropical variety with high yield and quality. Though this variety is cultivated in many parts of subtropical region, it is susceptible to the major sugarcane disease, red rot. The variety is being replaced by other varieties due to this problem. For imparting disease resistance, CoJ 64 was used as a parent in many crosses with red rot resistant varieties. Although new varieties are identified from the resulting seedling population, none combined the desirable traits of CoJ 64. It was in this context CoJ 64 was considered as a variety for sexual improvement through tissue culture. The somatic chromosome number of this variety is 2n = 108.

Callus culture

Callus culture was initiated from the innermost leaves and leaf sheaths surrounding the apical meristem of 6 months old field grown CoJ 64. The young leaf bits of about 0.5 x 0.5 cm were inoculated into 250 ml bottle with 20 ml MS basal medium (Murashige and Skoog, 1962) supplemented with 100 mg/L mesoinositol, 3 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 10% by volume coconut milk and 20 gm/L sucrose. The medium was solidified with 0.8% agar (Hi media). The pH was subjected to 5.8. For the induction of callus the cultures were inoculated in dark. The callus was subcultured three to four times at 15 days interval.

Plant regeneration and field analysis of somaclones

The friable calli were transferred to solid differentiation medium and kept in light for 8 h. The plantlet development and growth were obtained in MS medium with additions: 10 mg/L NAA, 2 mg/L kinetin, 0.5 mg/L thiamine HCl, 0.5 mg/L pyridoxine HCl, 100 mg/L mesoinositol and 20 mg/L sucrose.

Shoot buds differentiated from the calli were subcultured biweekly. Well grown shoots were separated and kept for rooting in half strength MS medium with 5.0 mg/L kinetin and 50 mg/L sucrose. Rooted plantlets were transplanted to polytene bags filled with sand, soil and farm yard manure in 1:1:1 ratio.

Plants were covered with plastic sheets to maintain humidity and kept in the green house for 50 to 60 days and then transferred to the field. One hundred and fifty six somaclones were established in the field and grown to maturity. These were scored for morphological characters and also for brix and sucrose percent. On the basis of apparent morphological variation including stalk and leaf characters, 42 clones were selected and forwarded to next vegetative generation through stem cuttings and further evaluation was taken in subsequent three years.

For evaluation, the morphological data were collected at ten months of age in each year. Crop characters measured were number of millable canes (NMC), stalk length, stalk diameter, leaf length, leaf width, brix percent, sucrose percent and purity. Three measurements were taken in each genotype for all the characters except NMC.

Cytological analysis

Cytological analysis was carried out in all the selected clones and control to determine the somatic chromosome number. Sets of each clone were placed in pots and 1 cm long root tips were pre treated in a saturated solution of α-bromonaphthaline for 2 h at 4ºC, washed in distilled water and fixed in 3:1 alcohol: acetic acid solution for overnight. After hydrolysis in 1 N HCl for 13 min at 60°C, the roots were stained in lueco basic fuschin and squashed in 1% acetocarmine. Chromosome number of the somaclone was ascertained from well spread metaphase plates.

Random amplified polymorphic DNA (RAPD) analysis of plants regenerated from embryogenic calli

Deoxyribonucleic acid (DNA) was extracted using the protocol of Walbot (1988). Sample for DNA extraction were the control, which is propagated through sets and seven randomly selected somaclones of CoJ 64. Genomic DNA was quantified spectrometrically (Sambrook et al., 1989).

Polymerase chain reaction (PCR) amplification was done as described by William et al. (1990) using RAPD primers (Operon Technologies, Alameda, USA). Twenty operon primers were screened with 2 samples and out of this, 12 primers were selected for the study. The selected primers were OPU-02, OPC-16, OPM-06, OPH-04, OPH-03, OPN-15, OPM-05, OPC-15, OPC-04, OPH-05, OPC 19 and OPC 10. PCR was performed in 15 ml reaction. 12 ng of the template DNA was amplified with one unit of taq DNA polymerase (0.3 µl) in a 15 µl reaction mixture containing 1 x PCR buffer (1.5 µl), 0.2 mM of dNTPs (1.2 µl) and 7.5 ng of random primer.

Amplification was performed in a BioRad PTC 200 Thermal Cycler System and was initiated by a denaturation of 3 min at 95°C followed by 44 cycles of 94°C for 1 min, 37°C for 1 min, 72°C for 2 min. The amplification was completed with 1 cycle of 7 min at 72°C.

RESULTS AND DISCUSSION

Production of somaclones and field analysis

Leaf bits of the variety produced good friable calli in callus induction medium and these calli differentiated in sold differentiation medium. The plants were hardened and after 60 days they were transferred to the field (Figure 1A to D). Out of 160 somaclones, 156 survived in the field. All the plants were visually graded at 10 months of age. The range values for different characters were given in Table 1. On the basis of apparent morphological variation and sucrose, 42% somaclones were selected for further evaluation.

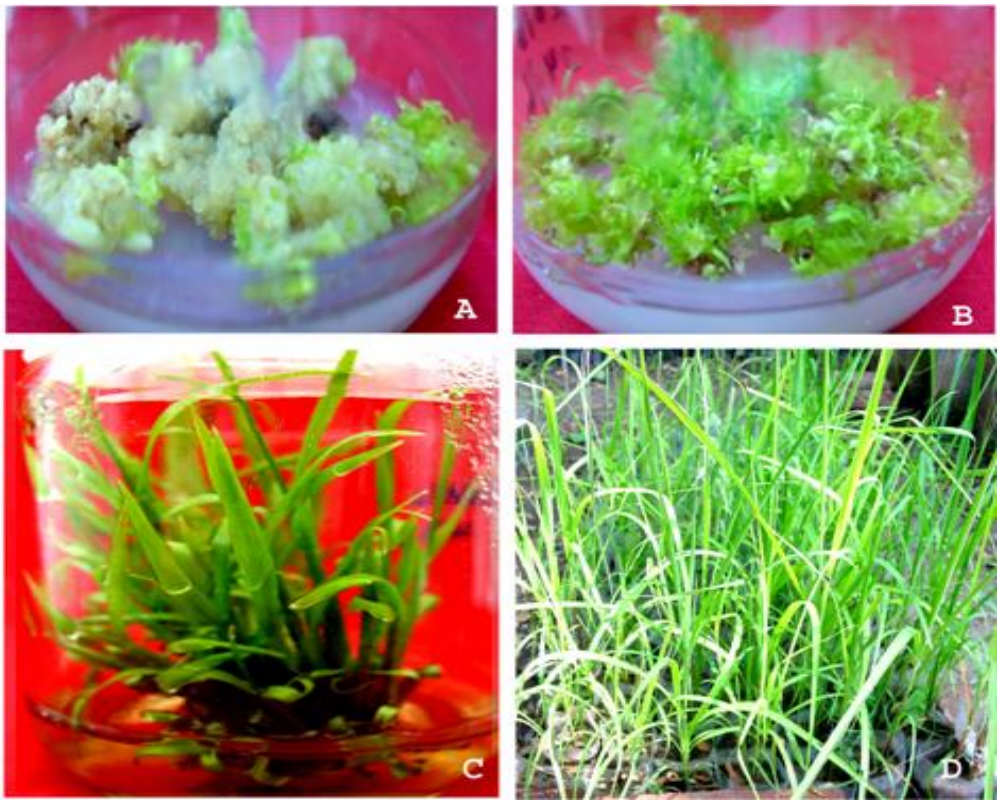

Figure 1. Callus culture of CoJ 64; (A) Callus formation from young leaf tissues (B) Callus regeneration (C) Shoots obtained from calli and (D) hardened somaclones in polybags.

Table 1. Range in values for the characters studied in 156 somaclones of CoJ 64.

Character	Range	Control
Stalk length (cm)	162.0 - 258.0	170.0
Stalk diameter (cm)	1.9 - 2.6	2.3
No. of internodes	16.0 - 38.0	35.0
Leaf length (cm)	81.3 -126.7	90.0
Leaf width (cm)	2.63 - 4.17	3.0
Brix	18.3 - 22.0	20.8
Sucrose (%)	16.5 - 20.78	19.5
NMC	27.0 - 51.0	28.0

During two constitutive years the 42 somaclones were phenotypically evaluated. Phenotypic variation was observed among the somaclones. The clones showed stable somaclonal variation after two clonal generations when compared with the control. Simple correlations among most characters measured were similar to other reported sugarcane populations (Table 2). Significant negative correlations were found between refractometer solids (HR brix), number of internodes and stalk length (Brown et al., 1969).

The number of internodes was significantly correlated with the leaf characters like leaf width and also stalks length. No significant negative correlation was found between stalk number and diameter although this inverse relationship had been reported in other sugarcane populations (Meyer et al., 1974; Milligan et al., 1990). The results indicated that the variation among the somaclones was repeatable over asexual cycles and were stable.

Molecular analysis of somaclones

Out of selected 46 somaclones, seven clones were selected randomly and the sensitivity of RAPD technique

Table 2. Simple correlations of agronomic characters.

Character	Stalk diameter	Stalk length	No. of internodes	Leaf length	Leaf width	HR Brix%
Stalk diameter	1	-0.041	0.117*	-0.127*	0.061	-0.068
Stalk length	-0.041	1	0.489**	0.328**	0.184**	-0.114
No. of internodes	0.117*	0.489**	1	0.269**	0.216**	-0.210**
Leaf length	-0.127*	0.328**	0.269**	1	0.363**	-0.085
Leaf width	0.061	0.184**	0.216**	0.363**	1	-0.123*
HR brix (%)	-0.068	-0.114	-0.210**	-0.085	-0.123*	1

*Correlation is significant at the 0.05 level (2-tailed). **Correlation is significant at the 0.01 level (2-tailed).

Table 3. DNA polymorphisms detected in somaclones of CoJ 64.

Primer code	Primer	Sequence (5' -3')	Total no. of band	Monomorphic band	Polymorphic band	Polymorphic percentage
P1	OPU 02	CTGAGGTCTC	3	1	2	66.7
P2	OPC 16	CACACTCCAG	4	1	3	75.0
P3	OPM 06	CTGGGCAACT	3	1	2	66.7
P4	OPH 04	GGAAGTCGCC	5	1	4	80.0
P5	OPH 03	AGACGTCCAC	7	-	7	100.0
P6	OPN 15	CAGCGACTGT	4	1	3	75.0
P7	OPM 05	GGGAACGTGT	5	1	4	80.0
P8	OPC 15	GACGGAGCAG	6	1	5	83.3
P9	OPC 04	CCGCATCTAC	6	1	5	83.3
P10	OPH 05	AGTCGTCCCC	4	-	4	100.0
P11	OPC 19	GTTGCCAGCC	9	-	9	100.0
P12	OPC 10	TGTCTGCGTG	6	-	6	100.0

tested for detecting polymorphism among the somaclones and control plant (Table 3). Of the 20 primers initially used for screening, 12 were selected for the study (Figure 2A to D). Each primer produced 3 to 9 bands, making a total of 3 to 9 bands making a total of 62 scorable bands. An average of 5 bands was scored per primer. These primers were sufficient to reveal multiple polymorphisms between the tested somaclones.

Majority of the primers produced a distinct pattern of amplification products for each clones. In OPC 04 and OPM 04, all the seven somaclones showed similar pattern of banding as control plant. Polymorphism was maximum in primer OPH 03. Here, addition and deletion of fragments were observed among the seven somaclones studied. This deletions or insertions in amplified regions or base changes altering primer binding sites will result in polymorphism. The polymorphism in these plants with its primer was consistent in another DNA extraction after 6 months. The primer that did not produce scorable markers either produced faint or non consistent amplification products, or no amplification product. Among the somaclones tested, 1 and 4 regenerants showed deletion and addition of parental bands in all most all the primers used.

This study clearly demonstrates the utility of RAPD markers in assessing the degree of somaclonal variation at genotypic level. A significant higher level of genotypic polymorphism was detected than expected based on phenotypic assessment.

The RAPD analysis mostly reveals repetitive sequences. Hence it is possible to assume that the rearrangements in repetitive sequences might be the reason for the difference between the control and somaclones obtained from callus cultures. Detailed analysis of these sequences will provide for a better understanding of the nature and mechanism of somaclonal variation in plants (Osipova et al., 2001).

Cytological studies

In any assessment of somaclonal variation, it is important to consider the cytogenetic stability of variant clones, particularly in a polyploid crop such as sugarcane (x = 8/10) in which eight complimentary set of chromosomes provide a degree of buffering that may enable a certain amount of cytogenetic abnormality to be tolerated. Aneuploidy is a common mechanism in progenies

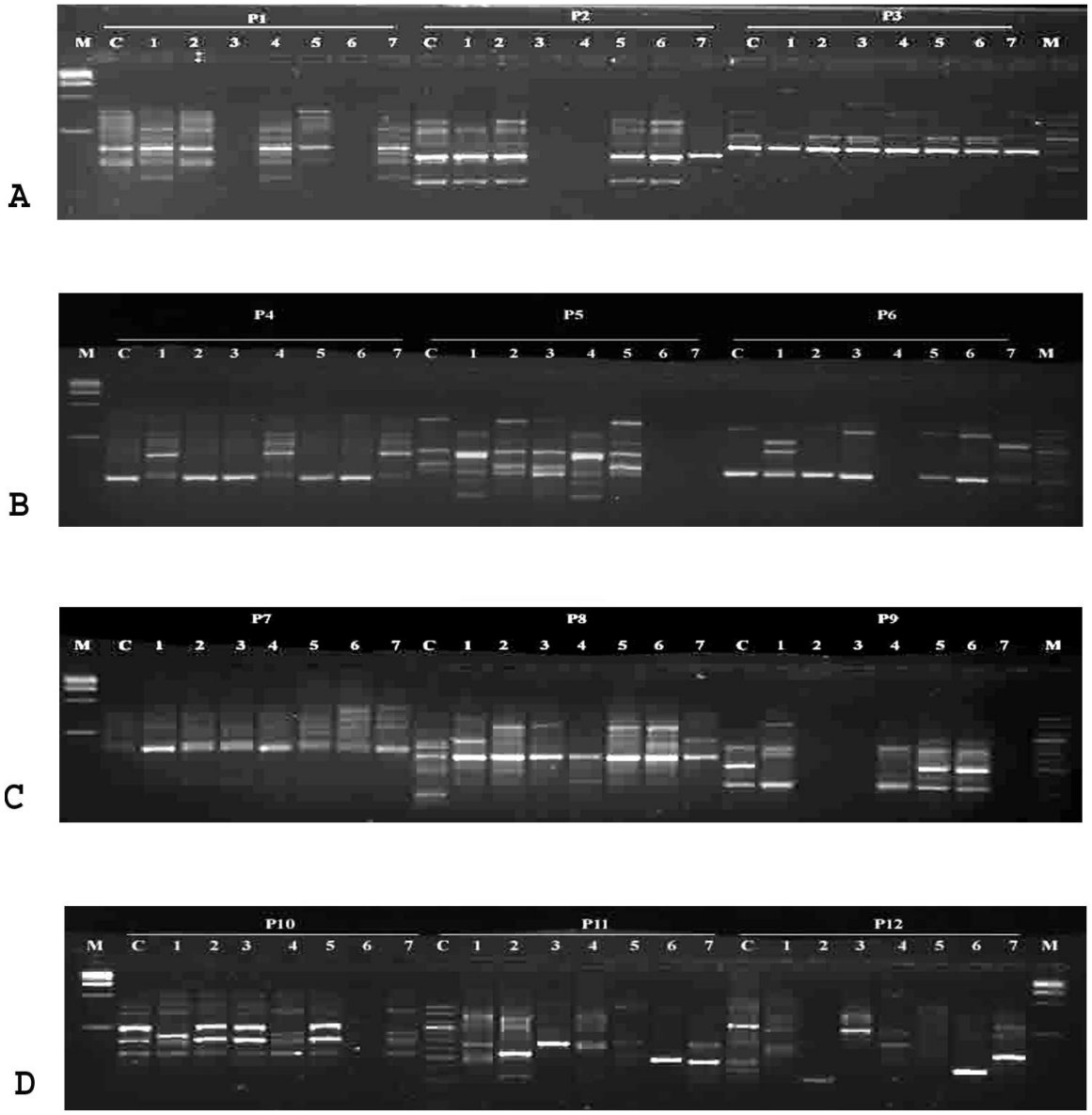

Figure 2. A to D Gel electrophoresis of RAPD fragments obtained in control plant (C-lane 2) and somaclones of CoJ 64 (1 to 7) using 12 primers (P1 to P12).

obtained through biparental cross.

In the present study, author investigated cytological variation in the plants regenerated through somatic embryogenesis (Figure 3A to D). The cytological investigations of 42 somaclones showed variation in chromosome number. However, a majority of the clones showed normal diploid number 2n = 108 (Table 4). Apart from the addition or deletion of the chromosomes morphologically altered chromosomes such as fragmented chromosomes were observed among the somaclones, but were not found in the control plant.

It was reported that the chromosomal variations shown by the cultured cells may be due to the mixoploid nature of the source (explant) used or due to the culture conditions (Phillips et al., 1994). Sometimes these variations in chromosome structure and number disturbs the physiological and genetic balance of the callus leading to a loss in capacity to regenerate plants (Torrey, 1967; Singh, 1986).

Somaclonal variation might have a more significant role as an addition to current plant breeding techniques if it is found to be different from the variation that can be produced by existing procedures. The reported studies here with this sugarcane variety did not reveal any apparently novel changes In this study, a method has been described for producing sugarcane regenerates

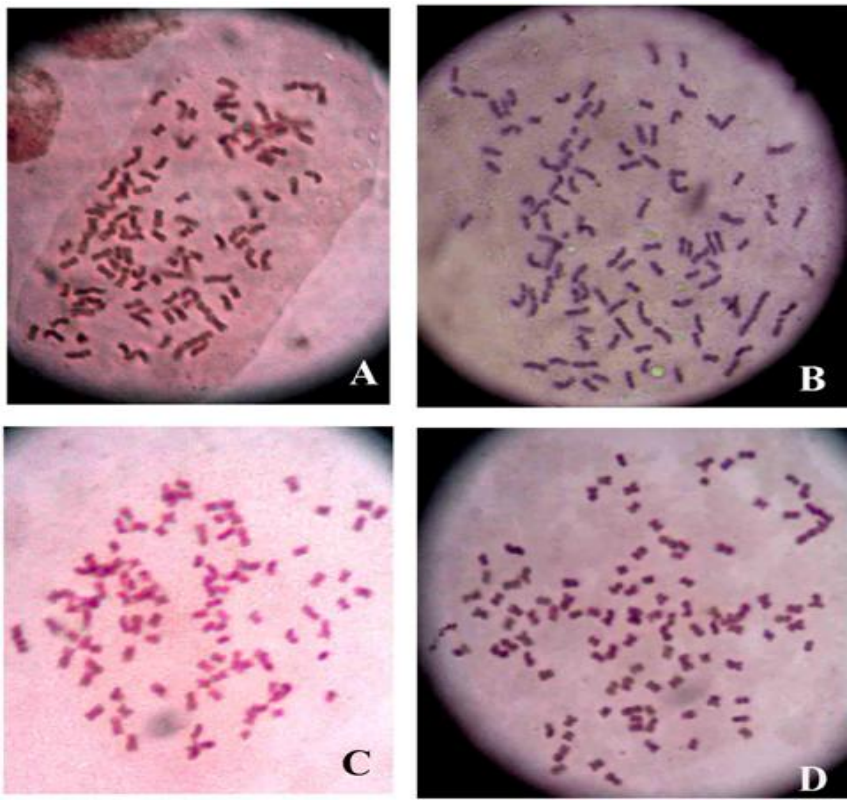

Figure 3. Root tip squash of somaclone of CoJ 64; (A) Somaclone with 2n = 110 (B and D) Somaclones with 2n = 108 (C) Somaclone with 2n = 100.

Table 4. Chromosome number of the somaclones of CoJ 64.

Cytotype	No. of somaclone	Chromosome number (2n) observed
2n = 108	27 (64.3%)	108
2n = <108	11 (26.2%)	100, 103, 99, 96, 102
2n = >108	4 (9.5%)	110

from callus cultures. The plants exhibited variation at the phenotypic, cytological and molecular levels and this assessment of tissue culture plants done during three consecutive years ruled out the chance of resulting variation due to. environment. If more information are obtained on the mechanisms which are responsible for genetic changes in culture, and factors which influence it, this should enable greater control of somaclonal variation, either to reduce it, or to direct it in a particular way, depending on the requirements from the regeneration system.

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