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Analysis of Callus Generated Somoclonal Variation in Proso Millet (*Pannicum milliaceum* L.) Through Molecular Markers

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Analysis of somaclonal variation by molecular markers allows precise varietal characterization. In this study, somaclonal variation of two local cultivars of proso millet was developed through callus culture and Inter Simple Sequence Repeat (ISSR) markers were used to detect somaclonal variation within somaclones and between somaclones and their parent cultivar since these markers are more reliable and detect the polymorphism at individual level. Thirteen ISSR primers were used per cultivar which has generated a total of 158 alleles in Sakhroli while 170 alleles in Asond cultivar with an average of 12 and 13 alleles in Sakhroli and Asond cultivar, respectively. The polymorphic information content (PIC) in Sakhroli cultivar ranged from 0.14 to 0.88 with an average of 0.50 while in Asond cultivar, it was ranged from 0.39 to 0.89 with an average of 0.69 The unweighted pair group method of arithmetic means (UPGMA) grouped 22 somaclones with their control parent of Sakhroli cultivar into two main clusters which were further divided into two sub-clusters. Similarly, UPGMA grouped 16 somaclones with its control parent of Asond cultivar into two main clusters which were further divided into two sub-clusters.

(***Key words:*** *ISSR markers, Polymorphism, Proso millet, Somaclonal variation,*)

Proso millet (*Pannicum milliaceum* L.) is of ancient cultivation and probably domesticated in Central and Eastern Asia. In Maharashtra, the largest area is found in coastal region comprising of Raigad, Thane, Sindhudurg and Ratnagiri districts. It is highly self-pollinated, cleistogamous plant having tiny florets, densely present on inflorescence and anthesis takes place during midnight. The number of superior genes present in different genotypes could not be brought together in one genotype through traditional breeding method because cumbersome breeding procedure. Hence, the only possibility to get rid over this is to adopt biotechnological tool *i.e.* callus culture for crop improvement in proso millet for yield in general and nutritional characters in particular.

The ISSR (Inter Simple Sequence Repeat) technique is a powerful, rapid, simple, reproducible and inexpensive way to assess genetic diversity or to identify closely related cultivars in many species, including fruit trees (Moreno *et al*., 1998). Assessment of somaclonal variation using DNA markers is one of the key tools of crop improvement and germplasm conservation. Several reports are available assessing the genetic diversity in proso millets using DNA based molecular markers namely RAPD (Fakrudin *et al*., 2004, Salimath *et al*., 1995) and ISSR (Dellaporta *et al*., 1983).

The use of molecular markers allows the direct assessment of genotypic variation at the DNA level. Marker analysis helps to understand the genetic makeup of the accessions and also make it possible to analyze the global organization of genetic diversity within a species. In the present study, callus derived somaclones of prosso millet were analysed for its variability.

MATERIALS AND METHODS

Plant material

In the present investigation 22 somaclones of Sakhroli and 16 somaclones of Asond genotypes of proso millet which were available at Plant Biotechnology Centre, College of Agriculture, Dapoli were analyzed. All the 22 somaclones with its control parent of Sakhroli cultivar and 16 somaclones with its control parent of Asond were grown in to the greenhouse. The leaf samples for DNA isolation were collected from 15 days old somaclones and their parental cultivars.

DNA extraction

The genomic DNA was isolated from the young newly flushing leaves of somaclones and their parents by following the protocol of Doyle and Doyle (1990) *i.e.* Rapid method with the slight modifications of buffer composition and concentration. Purification of DNA was done to remove RNA, proteins and polysaccharides which were the major contaminants. Concentration of DNA in the sample was determined after agarose gel electrophoresis with standard DNA *i.e.,* standard DNA

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ladder on 0.8 per cent agarose gel and by comparison of the intensity of staining with ethidium bromide.

PCR and ISSR analysis

PCR (Eppendorf) amplification reactions were performed with ISSR primers (Sigma). A total of 16 ISSR primers were subsequently used for PCR amplification (Table 1). For the ISSR analysis, gradient PCR amplification of gene specific primers was carried out so as to determine the annealing temperature of each primer. PCR reaction was performed in 10 μl reaction mixture consisting 0.25 μl of 3U Taq DNA polymerase, 1.25 μl of 10x Taq assay buffer with 0.25 μl of 15 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, 0.5 μl of 5 Pmole ISSR primer , 1 µl of template DNA and remaining volume was made by using sterile water. The amplification profile for ISSR consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles comprising of a denaturation step at 94°C for 45 sec, each primer has shown different annealing temperature for 1 min and an extension step at 72°C for 30 sec. The cycling program was terminated by a final extension step at 72 °C for 7 min. The amplified products in ISSR reaction were separated by electrophoresis in 2 per cent agarose gel (Banglore, Genei), containing ethidium bromide in 1x TAE Buffer (pH 8.0) and separation was carried-out by applying constant voltage of 60 volts for 1 hour.

The gels were photographed under UV light using Pentax K 312 nm camera. The images of gel was taken by the documentation systems (Uvi-Tech, Fire reader, Cambridge, England) and saved in computer for further analysis.

Data analysis

Based on the banding pattern obtained, the polymorphism percentage was calculated with different primers. The amplified products for each primer will be scored as 1 for presence of the band and 0 for the absence of the band to create a binary data matrix. Pair-wise similarity matrices were generated by Jaccard's coefficient of similarity by using MVSP-A Multivariate Statistical Package_5785 (Version 3.1). Distance matrix and dendrogram was constructed based on diversity coefficient generated from pooled data by using unweighted pair group method of arithmetic means (UPGMA), a computer programme for distance estimation. Other parameters computed were,

$$\text{Per cent polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

RESULTS AND DISCUSSIONS

The present investigation was carried out with an objective to analyze callus generated somaclonal variation in proso millet through molecular markers.

Table 1. *Details of various ISSR primers used for PCR amplification in proso millet somaclones*

Sr. No.	Primer	Primer sequence	Range of temperature	Annealing temperature	GC Content
		(5' – 3')	(°C)	(°C)	(%)
1.	UBC-807	AGAGAGAGAGAGAGAGT	42-47	45.5	47.01
2.	UBC-809	AGAGAGAGAGAGAGAGT	50-55	52.8	47.1
3.	UBC-811	GAGAGAGAGAGAGAGAC	50-55	52.8	52.9
4.	UBC-816	CACACACACACACACAT	48-53	51.1	47.1
5.	UBC-822	TCTCTCTCTCTCTCTCA	50-55	52.8	47.01
6.	UBC-825	ACACACACACACACACT	48-52	50.4	47.1
7.	UBC-827	ACACACACACACACACG	52-57	54.9	52.9
8.	UBC-828	TGTGTGTGTGTGTGTGA	51-56	53.2	47.1
9.	UBC-834	AGAGAGAGAGAGAGAGT	42-48	45.4	47.1
10.	UBC-836	AGAGAGAGAGAGAGAGC	50-55	52.6	52.9
11.	UBC-841	GAGAGAGAGAGAGAGAC	52-56	54.8	52.9
12.	UBC-844	CTCTCTCTCTCTCTCTC	43-48	46.5	52.9
13.	UBC-848	CACACACACACACACAT	52-56	54.8	47.1
14.	UBC-857	ACACACACACACACYG	55-60	57.1	42.1
15.	UBC-878	GGATGGATGGAT	28-33	29.00	50.0
16.	UBC-891	TGTGTGTGTGTGTG	78-83	81.1	50.0

Somaclonal variability is a source of useful genetic variations; during selection, it makes it possible to select *in vitro* plant material exhibiting certain valuable agricultural traits. Somaclonal variability can be regarded as an efficient tool for breeders, because combining in vivo cultivation methods and classic breeding methods considerably accelerates creation of new plant varieties (Baer *et al.*, 2007). In the present study 22 somaclones of Sakhroli cultivar and 16 somaclones of Asond cultivars with the control parents of both cultivars of proso millet characterized on the basis of molecular markers. Molecular markers have been proven to be powerful tools in the assessment of somaclonal variation and in the elucidation of relationships within the species by using ISSR markers (Federic *et al*., 2007; Mahdy, 2011)

Polymorphism percentage

All 22 somaclones of sakhroli and 17 somaclones of Asond cultivar were successfully amplified with their parents with thirteen ISSR primers for each cultivar (Table 2 and 3). In Sakhroli cultivar all 11 ISSR primer were found to be polymorphic and two primers namely; UBC-807 and UBC-878 were monomorphic. While in Asond cultivar only UBC-816 are observed as a monomorphic. The overall average size of amplified products ranged from 380 to 1173 bp in Sakhroli and 300-1300 bp in Asond. A total of 158 and 170 alleles were obtained using 13 ISSR primer in Sakhroli and Asond with an average of 12 and 13 alleles per primer, respectively.

The present study utilized 22 somaclones of Sakhroli and 16 Somaclones of Asond cultivar of proso millet with its control parent for ISSR analysis with 13 random primers. In Sakhroli somaclones, the primers UBC-816, UBC-827 (Plate 1), UBC-841, UBC-848, UBC-857 and UBC-891 produced high degree of polymorphism with an average of 64.9 per cent while in Asond somaclones, the primers UBC-809, UBC-811, UBC-822, UBC-827, UBC-834, UBC-836, UBC-848, UBC-857 and UBC-891 (Plate 2) produced high degree of polymorphism with an average of 85.40 per cent. Hemaprabha *et al.* (2013) recorded similar results in analysis of genetic diversity produced in regenerated plant by anther culture.

Polymorphism information content

The Polymorphism Information Content (PIC) values were calculated to find out the efficiency of primers in distinguishing individual cultivars. The PIC values of primers ranged from 0.14 in primer UBC- 844 to 0.88 in primer UBC- 827 of Sakhroli cultivar while the PIC value of primer ranged from 0.39 in primer UBC-878 to 0.89 in primer UBC- 891. The similar result was obtained by the (Prabhu and Ganeshan, 2013) that is the PIC value was highest for the primer ISSR 44 (0.761). The high PIC value obtained in the present investigation might be due to high genetic diversity among the proso millet cultivar.

Table 2. *Molecular polymorphism, PIC values, and size of loci revealed by ISSR primers in Sakhroli cultivar*

Sr. No.	Primer	Amplification Range (bp)	Per cent Polymorphism	No. of Alleles	PIC
1	UBC-807	300-1450	0.00	2	0.00
2	UBC-811	250-1350	60.60	16	0.52
3	UBC-816	300-1000	100.00	14	0.73
4	UBC-827	250-1050	100.00	16	0.88
5	UBC-828	250-1600	53.06	15	0.40
6	UBC-834	250-1000	78.00	15	0.61
7	UBC-836	300-900	33.30	8	0.25
8	UBC-841	50-1000	100.00	17	0.78
9	UBC-844	450-1000	19.30	7	0.14
10	UBC-848	300-850	100.00	9	0.58
11	UBC-857	450-1950	100.00	20	0.86
12	UBC-878	1850-1900	00.00	1	0.00
13	UBC-891	250-1650	100.00	18	0.83
	Total	–	–	**158**	–
	Average	**380-1173**	**64.90**	**12.15**	**0.50**

Table 3. *Molecular polymorphism, PIC values and size loci revealed by ISSR primers in Asond cultivar*

Sr. No.	Primer	Amplification Range (bp)	Per cent Polymorphism	No. of Alleles	PIC
1	UBC-807	300-1450	76.38	15	0.63
2	UBC-809	50-1100	100.00	16	0.86
3	UBC-811	250-1700	100.00	17	0.88
4	UBC-816	800-850	00.00	1	0
5	UBC-822	200-1800	100.00	16	0.67
6	UBC-825	300-1400	73.40	11	0.56
7	UBC-827	150-1100	100.00	17	0.83
8	UBC-834	400-1350	100.00	8	0.76
9	UBC-836	300-1050	100.00	13	0.79
10	UBC-848	200-1000	100.00	13	0.76
11	UBC-857	300-1600	100.00	16	0.79
12	UBC-878	350-900	60.46	7	0.39
13	UBC-891	300-1550	100.00	20	0.89
	Total	–	–	**170**	–
	Average	**300-1300**	**85.40**	**13.08**	**0.68**

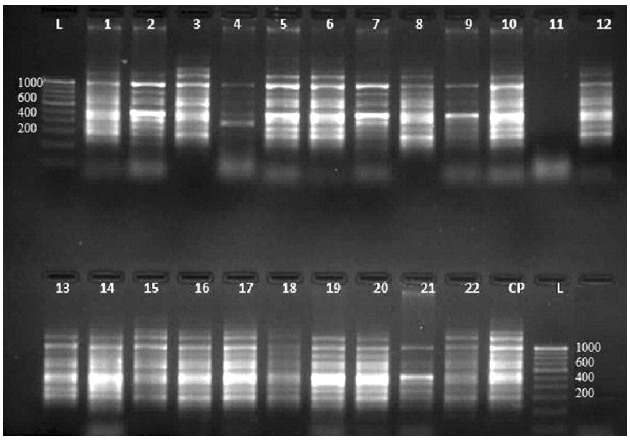

Plate 1. *ISSR profile of 22 somaclones of Sakhroli cultivar by using primer UBC-827* (L: 100 bp Molecular Ladder, Lane 1-22: Somaclones, CP: Control Parent)

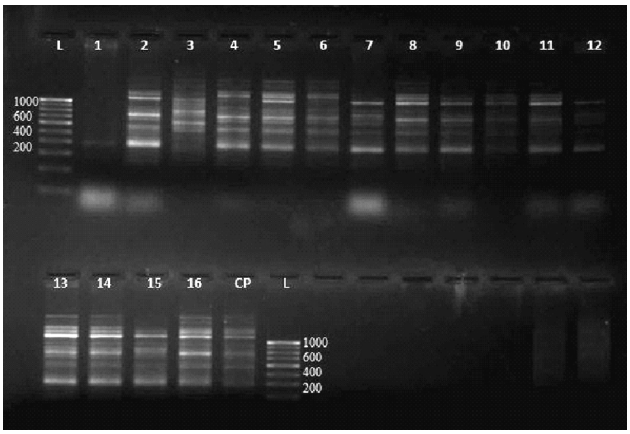

Plate 2. *ISSR profile of 16 somaclones of Asond cultivar by using primer UBC-891* (L: 100 bp Molecular Ladder, Lane 1-16: Somaclones, CP: Control Parent)

Jaccard's similarity coefficient and cluster analysis

The Jaccard's similarity coefficient values among these Sakhroli cultivars are obtained, the pair wise similarity values ranged from 0.178 to 0.667 (Table 4). Maximum similarity value of 0.667 was noticed between SSCN-5 and SSCN-6. Minimum similarity value of 0.178 was observed between SSCN-8 and control parent while among these Asond cultivar, the pair wise similarity value ranged from 0.120 to 536 (Table 5). Maximum similarity value of 0.536 was noticed between ASCN-16 and control parent and minimum similarity value of 0.120 was noticed between ASCN-9 and ASCN-1. Similar observations were also recorded by (Hemaprabha *et al*., 2013) carried out the study on genetic diversity analysis anther culture derived rice plant using RAPD and ISSR Markers.

Marker alleles were converted to binary scores based on their presence or absence as 1 and 0, respectively. UPGMA cluster analysis was performed using Jaccard's similarity coefficient matrices calculated from ISSR markers to generate a dendrograms for 22 somaclones of Sakhroli and 16 somaclones of Asond with their control parent (Fig. 1 and 2). The UPGMA grouped 22 somaclones with their control parent of Sakhroli cultivar into two main clusters which were further divided into two sub-clusters as well as the UPGMA grouped 16 somaclones with its control parent of Asond cultivar into two main clusters which were

Table 4. *Somaclonal variation based on ISSR pooled over the 13 primers in 23 Genotype of proso millet*

Similarity matrix	SCN-1	SCN-2	SCN-3	SCN-4	SCN-5	SCN-6	SCN-7	SCN-8	SCN-9	SCN-10	SCN-11	SCN-12	SCN-13	SCN-14	SCN-15	SCN-16	SCN-17	SCN-18	SCN-19	SCN-20	SCN-21	SCN-22	Control parent
SCN-1	1																						
SCN-2	0.483	1																					
SCN-3	0.45	0.471	1																				
SCN-4	0.397	0.407	0.481	1																			
SCN-5	0.338	0.318	0.467	0.483	1																		
SCN-6	0.338	0.359	0.419	0.459	0.667	1																	
SCN-7	0.314	0.429	0.397	0.547	0.603	0.603	1																
SCN-8	0.348	0.397	0.39	0.456	0.469	0.649	0.611	1															
SCN-9	0.282	0.362	0.379	0.473	0.415	0.484	0.574	0.536	1														
SCN-10	0.32	0.338	0.354	0.413	0.429	0.429	0.525	0.593	0.484	1													
SCN-11	0.273	0.358	0.327	0.451	0.417	0.371	0.56	0.463	0.54	0.574	1												
SCN-12	0.381	0.389	0.407	0.426	0.375	0.294	0.328	0.281	0.29	0.294	0.327	1											
SCN-13	0.282	0.275	0.348	0.324	0.365	0.384	0.306	0.284	0.292	0.295	0.284	0.30	1										
SCN-14	0.333	0.356	0.397	0.344	0.348	0.348	0.323	0.299	0.288	0.31	0.322	0.39	0.621	1									
SCN-15	0.275	0.288	0.351	0.322	0.29	0.29	0.281	0.239	0.266	0.271	0.345	0.37	0.607	0.519	1								
SCN-16	0.246	0.276	0.364	0.31	0.261	0.243	0.27	0.209	0.254	0.243	0.333	0.38	0.467	0.569	0.65	1							
SCN-17	0.338	0.364	0.382	0.283	0.275	0.294	0.266	0.262	0.25	0.313	0.304	0.33	0.508	0.62	0.51	0.563	1						
SCN-18	0.299	0.316	0.382	0.4	0.294	0.275	0.35	0.281	0.333	0.313	0.404	0.40	0.534	0.588	0.63	0.596	0.52	1					
SCN-19	0.299	0.313	0.309	0.343	0.329	0.329	0.362	0.301	0.329	0.347	0.365	0.32	0.397	0.492	0.36	0.375	0.459	0.483	1				
SCN-20	0.394	0.404	0.373	0.323	0.292	0.292	0.323	0.279	0.269	0.329	0.368	0.35	0.382	0.509	0.41	0.404	0.528	0.528	0.649	1			
SCN-21	0.37	0.333	0.309	0.364	0.263	0.263	0.362	0.267	0.329	0.295	0.344	0.30	0.342	0.424	0.36	0.397	0.391	0.459	0.569	0.516	1		
SCN-22	0.279	0.339	0.31	0.262	0.239	0.239	0.306	0.224	0.29	0.275	0.327	0.33	0.369	0.421	0.45	0.563	0.49	0.462	0.508	0.473	0.561	1	
Control parent	0.3	0.317	0.333	0.306	0.227	0.195	0.269	0.178	0.235	0.227	0.283	0.42	0.274	0.393	0.30	0.436	0.356	0.404	0.409	0.466	0.476	0.481	1
	SCN-1	SCN-2	SCN-3	SCN-4	SCN-5	SCN-6	SCN-7	SCN-8	SCN-9	SCN-10	SCN-11	SCN-12	SCN-13	SCN-14	SCN-15	SCN-16	SCN-17	SCN-18	SCN-19	SCN-20	SCN-21	SCN-22	Control parent

No. 1 to 22: Somaclones and No. 23: Sakhroli (control)

Table 5. *Somaclonal variation based on ISSR pooled over the 13 primers in 23 Genotype of proso millet*

Similarity matrix	SCN-1	SCN-2	SCN-3	SCN-4	SCN-5	SCN-6	SCN-7	SCN-8	SCN-9	SCN-10	SCN-11	SCN-12	SCN-13	SCN-14	SCN-15	SCN-16	Control parent
SCN-1	1																
SCN-2	0.316	1															
SCN-3	0.307	0.256	1														
SCN-4	0.235	0.389	0.307	1													
SCN-5	0.218	0.338	0.274	0.377	1												
SCN-6	0.277	0.432	0.268	0.432	0.384	1											
SCN-7	0.215	0.297	0.175	0.263	0.264	0.275	1										
SCN-8	0.247	0.213	0.203	0.247	0.265	0.329	0.381	1									
SCN-9	0.12	0.273	0.206	0.254	0.317	0.268	0.356	0.389	1								
SCN-10	0.16	0.205	0.165	0.19	0.254	0.282	0.25	0.288	0.322	1							
SCN-11	0.159	0.218	0.177	0.203	0.2	0.263	0.282	0.246	0.295	0.328	1						
SCN-12	0.158	0.222	0.132	0.128	0.186	0.19	0.235	0.197	0.2	0.171	0.277	1					
SCN-13	0.195	0.195	0.171	0.225	0.192	0.195	0.175	0.187	0.206	0.165	0.208	0.303	1				
SCN-14	0.173	0.218	0.177	0.218	0.2	0.202	0.182	0.162	0.179	0.156	0.184	0.361	0.431	1			
SCN-15	0.181	0.164	0.186	0.214	0.176	0.197	0.191	0.226	0.131	0.179	0.194	0.259	0.407	0.481	1		
SCN-16	0.188	0.188	0.192	0.218	0.2	0.188	0.213	0.178	0.197	0.203	0.25	0.258	0.388	0.406	0.481	1	
Control parent	0.152	0.213	0.219	0.23	0.194	0.213	0.225	0.123	0.25	0.164	0.178	0.215	0.348	0.284	0.357	0.536	1
	SCN-1	SCN-2	SCN-3	SCN-4	SCN-5	SCN-6	SCN-7	SCN-8	SCN-9	SCN-10	SCN-11	SCN-12	SCN-13	SCN-14	SCN-15	SCN-16	Control parent

No. 1 to 16: Somaclones and No. 17: Asond (control)

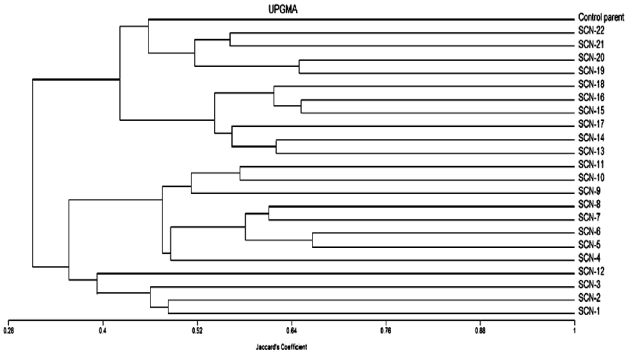

Fig. 1. *Dendrogram constructed using Jaccards Similarity Coefficient of Sakhroli somaclones*

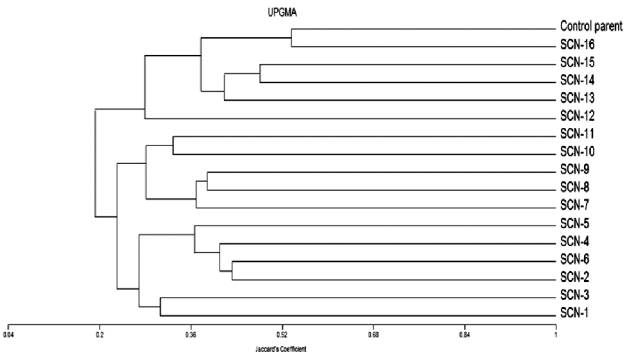

Fig. 2. *Dendrogram constructed using Jaccards Similarity Coefficient of Asond somaclones*

further divided into two sub-clusters. In the Asond cultivar it was observed that, the somaclone ASCN-12 occupied a unique position and was most diverse from rest of 15 somaclones of proso millet and one control parent line Asond. Similar results have been found by Gupta *et al*. (2012) for finger millet accessions in which the cluster one contain single cultivar PRM-1 and cluster two Contain two varieties PRM-701 and PRM-801, respectively based on ISSR analysis.

CONCLUSION

Somaclonal variation occurs due to tissue culture. The study indicated that ISSR markers are suitable for the assessment of Somaclonal variation among different somaclones of proso millet. The ISSR analysis revealed substantial polymorphism in proso millet. The results of the present study indicated the efficiency of ISSR markers in investigating genetic variability at molecular level, which is important for detecting variation of somaclones also for the identification of desirable somaclone and its utilization for further breeding programme. Such information may be useful for selecting the diverse parents and monitoring the genetic diversity periodically breeding for improvement of proso millet.

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