Production of sweet and high biomasssorghum lines with optimized cell wall components for increased biofuel bioconversion yield

Abstract

The current research focuses on the estimation of fibre content in sweet and highbiomass sorghum lines to assess biofuel productionthroughpartially replicatedexperimentaldesignbasedonBLUPsvalues; thisstudywascarriedoutforfourseasons(frompostrainy 2020 to rainy 2022) to developF_3 populations of sweet and high-biomass sorghumlines. The proximate fibre component analysis was done in the matured F_3populations bydryingtheplantsamplesandgrindingthemintoafinepowder.Fibrequalitycomponentssuchas cellulose, hemicellulose, acid detergent fibre (ADF), lignin (ADL), neutral detergent fibre(NDF), metabolizable energy (ME), nitrogen, in vitro organic matter digestibility (IVOMD),andash,wereassessedusingNear-InfraredReflectanceSpectroscopy(NIRS).ICSV18003hadthelowestADFpercentageat37.04%, whileitalsohadthehighestADLpercentageat3.84%.The ash percentage was 3.73% in SSV84 × N609. Moreover, the *bmr* introgressed lines of theF_3population exhibited higher levels of cellulose and hemicellulose, while lignin and ashcontent were decreased. This indicates that the *bmr6* and *bmr12* genes can be confidentlyutilized in sorghum breeding for bioenergy production, as they meet the requirements forbioethanolproduction.

Keywords:*bmr6*,*bmr12*, Proximatefibreanalysis,*Sorghumbicolor*(L.)Moench

Introduction

Oneofthebiggestproblems facingallnationsinthetwenty-firstcenturyistheworld'snever-endingneedforenergy.Thiscircumstancehasspurredthesearchforandimplementationofsustainableenergygenerationoptions and thegrowingconcernovercarbon emissions that cause global warming and the depletion of non-renewable fossil fuel sources(Sentanuhady *et al*. 2021). The viability of biofuels as a renewable energy source can dependon several variables, including the feedstock utilized in biofuel production, the economicinfrastructure, geographic location and climate. As a result, nations should developclimate-appropriateoil-producingcropsandsetupincentivesthatwouldbesimpletoimplementin theirexistinginfrastructure(Khan *et al*. 2021).

Sweet sorghum is an excellent choice for semi-arid and arid marginal regions becauseit withstands water stress. Its morphophysiological traits confer drought endurance (Zegada-Lizarazu & Monti, 2013), and the C_4 photosynthetic system facilitates adequate CO_2 fixationandextraordinarydrymatterbuild-up(Mastrorilli*etal*.,1999).Likemostotherlignocellulosicbiomasses, sorghum biomass contains about 20% lignin(Bout & Vermerris, 2003). High lignin content hinders biofuelsynthesisevenifitgivesthecellwallmechanicalstrength.Ligninremovalisexpensive,which challenges manufacturing 2G commercial biofuels. *Bmr6 and bmr12* play asignificantroleinthebiosynthesisofsorghumligninandhelp mutate C-to-Tatposition486relativeto the transcription's startingsite(Bout & Vermerris, 2003).

The hydrolysis of structural components ofbiofuelfeedstocks is influenced by such as arabinose to xylose ratio, penetration ofpretreatmentchemicals,crystallineversusamorphouscellulosestructure,contentsofligninandhemicellulose, and their distributions forconversiontofuels(Kurakake*etal*.,2001;Park*etal*.,2010;Vandenbrink *et al*., 2010). Significant variations are observed in the chemical composition ofsoluble and structural components between a non-sweet sorghum hybrid and a sweet sorghum cultivar(Dolciotti*etal*.,1998).Thenutritionalcompositionandfractionofcellwallsinfodderare directly correlated with the stage of maturity. The progressive maturation increases ligninlevels,reducingdigestibility,fodderquality,andanimalproductivity(Beck*etal*.,2013).ADF concentrationinforagesrepresentstherelativeamountofcelluloseandligninincellwalls.The NDF number represents the combined cell wall content, including ADF and hemicellulosecomponents.Ligninadherestofibrecomponents,suchascelluloseandhemicellulose, andisa physical obstacle to microbial enzyme degradation of plant cell walls (Salama & Nawar,2016).Severalstudieshavedocumentedtheelevatednutritionalcontent,dry-matterdigestibility(DMD),andorganic-matterdigestibility(OMD)inBMRsorghum(Li*etal*.,2015;Wahyono *et al*., 2019). Biomass sorghum with mutant genotypes exhibits low lignin - *bmr*(brown midrib) content and sensitive to photoperiod, which differ in terms of cell wallcomposition, fibre digestibility, followed by quantity and quality compared to conventionalmaterials(Sattleretal.,2010;Cherneyetal.,1991), are found to be promisingfortheproductionof 2Gethanol.Cellulosicethanolproductionrequiresthebreakdownoftheplantcellwallusingchemical andphysicalmethods.Usinglessresistantmaterialshelpsimprovethepretreatmentprocessforbreakingdownthelignocellulosicbiomass(Sattleretal.,2016;Oliveret al., 2005). The current research

focuses on developing F_3 populations through a partially replicated experimental design based on BLUPs values for proximate fibre contentanalysisof Sorghum [*Sorghum bicolor*(L.) Moench].

MaterialsandMethods

The present research was carried out in four seasons (from post-rainy 2020 to rainy 2022)to develop F_3populations of sweet and high-biomass sorghum lines and one-grain sorghumtype; for fibre **analysis study**, the experiments were conducted at the International CropsResearch Institute for the Semi-Arid Tropics (ICRISAT) in Patancheru, Hyderabad, India, atcoordinates17°27'Nand78°28'E.

Populationdevelopment

Parent selection and consecutive cross-breeding for developing F_1 hybrids was initiated in thefall of post-rainy 2020. Since sweet and high biomass sorghum lines are photoperiod lines,staggeredsowingwasfollowedtogetthepollenforon-timecrossingsfordevelopinghybrids.The reproductive organs (anthers) were removed manually from the recurrent parent plant,which was further pollinated with the pollen collected from *bmr*6 and *bmr*12 donor parents.The mature F_1 seeds were collected from the respective recurrent parent. In the second season(rainy,2021),fewF_1seedsweresown,andthepaniclesatthe base of the shoot wereselfedandadvanced for F_2populations for *bmr6* and *bmr12* hybrids for observing *bmr* segregationsphenotypically. During the third season (post-rainy, 2021), F_2populations were sowed toobserve *bmr* and wild type (WT) segregations phenotypically (the segregations for both *bmr6*and*bmr12 hybrids*wereobserved)toconfirmthehybridityforF_1throughphenotypicapproachandadvancedforF_3populations.Further,inthefourthseason(rainy2022),theF_3populationweresowed for Fiberanalysis and advancedfortheF$_4$population.

Table1-Listofparents usedforDevelopingF_3populations (Source: ICRISAT)

S.No.	Parent	*bmr*/WT	Trait
1	SSV84	WT	SS/HBM
2	ICSV15024	WT	HBM
3	ICSV18003	WT	SS
4	ICSB474	WT	SS/HBM
5	ICSV100324	WT	SS/HBM

6	ICSB38	WT	Graintype
7	N609	*bmr*	*bmr6*
8	ICSV101112	*bmr*	*bmr6*
9	ICSV101039	*bmr*	*bmr12*
10	N600	*bmr*	*bmr12*

SS:SweetSorghum,HBM:High-biomasssorghum, *bmr*-brownmidrib mutant, *Bmr*:wildtype*(non-Bmr)*

FiberComponentAnalysis

The F_3populations were sowed for Fiber analysis study by partially replicated experimentaldesignandadvancedfortheF$_4$populations.Theproximatefibrecomponentanalysiswa sdonein the matured F_3population by drying the plant samples and grounding them into a finepowder. Various fibre quality traits, including cellulose, hemicellulose, acid detergent fibre(ADF), lignin (ADL), neutral detergent fibre (NDF), metabolizable energy, nitrogen, in vitroorganicmatterdigestibility(IVOMD),andash,wereassessedusingNear-InfraredReflectanceSpectroscopy (NIRS) following the method adapted from Rivera-Burgos *et al*., (2019). Near-infrared spectroscopy (NIRS) is now a well-developed and mature technology that is used cost-effectively by thescientificcommunity.NIRSwaswidely appliedasarapidand**non-destructive** tool for several products, e.g., meat, fruit, or biomass feedstocks. The samples ofthe current study were analyzed by the International Livestock Research Institute (ILRI) intheirlabatthe InternationalCropsResearchInstitutefortheSemi-AridTropics,headquarters,India, using FOSS DS 2500 Forage analyzer and WinISI calibration software. The ILRIreported coefficients of determination (R^2) were satisfactory (i.e., 0.83, 0.91, 0.82, 0.91, and0.9,respectively, forNDF,ADF,ADL,ME,andIVOMD)and**moresignificant**thanpreviouslyreportedworks(e.g.,O tero*etal*.,2023).Thepresentinvestigationwasstudiedusing fibreanalysisbasedon thepartiallyreplicated BLUPvalues.

Table 2- ListofF3GenotypesusedforFiberDetergent Analysis(FDA)

S. No	F_3Populations	Noof Replications	S. No	F_3Populations	Noof Replications
1	ICSB38×N600	2	27	ICSB474×ICSV101112	1
2	ICSB38×N600	2	28	ICSB38×ICSV101039	1
3	SSV84 ×N609	2	29	SSV84 ×ICSV101112	1
4	SSV84 ×N609	2	30	SSV84 ×ICSV101112	1
5	ICSB38×N600	2	31	ICSV100324×ICSV101039	1
6	ICSB38×N600	2	32	SSV84 ×N609	1
7	ICSV100324 ×ICSV101039	2	33	ICSB474×ICSV101112	1
8	ICSV18003 ×N609	2	34	ICSB474×N600	1
9	ICSV18003 ×N609	2	35	ICSV15024 ×N609	1
10	SSV84 ×N609	2	36	ICSV15024×N609	1
11	SSV84 ×N609	2	37	ICSB474×ICSV101039	1
12	SSV84 ×ICSV101039	2	38	ICSB474×ICSV101039	1
13	ICSB38×ICSV101039	2	39	ICSB474×ICSV101112	1
14	SSV84 ×N609	2	40	ICSB474×ICSV101039	1
15	ICSB38×ICSV101039	1	41	ICSB474×ICSV101112	1
16	ICSV100324 ×ICSV101039	1	42	ICSB474×N600	1
17	SSV84×ICSV101039	1	43	SSV84 ×ICSV101039	1
18	ICSB474×N609	1	44	SSV84×ICSV101039	1
19	SSV84×ICSV101039	1	45	ICSB474×ICSV101039	1
20	ICSV100324 ×ICSV101039	1	46	ICSB474×ICSV101112	1
21	ICSV15024×ICSV101112	1	47	Check-1 ICSV18003	6
22	ICSV15024×ICSV101112	1	48	Check-2 ICSV100324	6
23	ICSB474×N609	1	49	Check-3SSV84	6
24	SSV84 ×ICSV101039	1	50	Check-4 ICSV15024	6
25	ICSB38×ICSV101039	1	51	Check-5ICSV101039*bmr*12	6
26	ICSB474×ICSV101112	1	52	Check-6N609*bmr*6	6
				Total	**96**

ToconfirmtheF$_1$hybrid,thesewere selfedforobtainingtheF$_2$population,segregationsinthe3:1ratiowereobserved,andthesegregatedF$_2$with *bmr*expressionswere

advanced for $F_3$by selfing F_2populations and evaluated by partially replicated design. Thepartially replicated experimental design (Cullis *et al*., 2006) was followed for F_3 populations,which consist of a total of 96 experimental units, with several treatments of 52, followed bysixchecks,eachcheckreplicatingsixtimesandreplicatedentrieswith14(eachentryreplicating two times) and un-replicated with 32 entries (replicating only once). There are 4 x4 blocks, resulting in 6 blocks; each block contains a set of 6 checks. The sub-blocks of 4 x 1allowed replicated entries to be evenly assigned within the columns (Gilmour *et al*., 1997).The partiallyreplicatedexperimentaldesignandF_3populationsarementioned in Figure1 below.

Fig.1-PartiallyReplicatedDesignforF_3Populations

ResultsPhenoty

picdata

The*bmr6*and*bmr12*hybridplantswerefoundtohavebrownmidribcolourinthematuredplant. However, the brown midrib phenotype was observed only in F_2 generations **but not inF_1,** confirming the recessive inheritance of the trait. In the F_2 population of *bmr*6, 18.5% ofplants were introgressed; in *bmr*12, 23.2% were introgressed. The phenotypic data and thepercentageof*bmr* inF_2Populations of*bmr*6 and*bmr*12 arementioned inTable 3.

Table3-PhenotypicdataforF₂Populations

bmr6							
S. No.	**CrossCombination**	**Stage**	***Bmr***	***bmr* plants**	**WT Plants**	**Total Plants**	**% of *bmr***
1	SSV84×N609	F_2	*bmr6*	7	15	22	31.8
2	SSV84×ICSV101112	F_2	*bmr6*	3	19	22	13.6
3	ICSV18003×N609	F_2	*bmr6*	3	19	22	13.6
4	ICSV15024×N609	F_2	*bmr6*	3	18	21	14.2

5	ICSV15024× ICSV101112	F_2	*bmr6*	4	17	21	19
6	ICSB474 ×N609	F_2	*bmr6*	3	19	22	13.6
7	ICSB474×ICSV101112	F_2	*bmr6*	5	16	21	23.8
			Total	28	123	151	18.5
bmr12							
1	SSV84×ICSV101039	F_2	*bmr12*	6	15	21	28.6
2	ICSB474×ICSV101039	F_2	*bmr12*	5	16	21	23.8
3	ICSB474 ×N600	F_2	*bmr12*	3	17	20	15
4	ICSV100324× ICSV101039	F_2	*bmr12*	5	16	21	23.8
5	ICSB38 ×N600	F_2	*bmr12*	5	16	21	23.8
6	ICSB38×ICSV101039	F_2	*bmr12*	5	16	21	23.8
			Total	29	96	125	23.2

BasedontheBLUPvalues,thestudyidentifiedthatthelowestmetabolizableenergy(ME)(MJ/kg) was found in ICSV15024 ×ICSV101112, followed by ICSB38 ×N600 with 5.74and 5.98, respectively. ICSV18003 had the lowest ADF percentage at 37.04%, while it alsohad the highest ADL percentage at 3.84%. The ash percentage was 3.73% in SSV84 × N609.Moreover, the *bmr* introgressed lines of the F_3 population exhibited higher levels of celluloseandhemicellulose,whileligninandashcontentweredecreased.Similarly,ashpercentagewaslowest in SSV84 × N609 at 3.73%, which was comparatively lesser when compared to the respectivedonor(5.60%)andthe recipient(5.33%)parent.CellulosepercentagewaslowestinSSV84× N609 with 32.59 %, and ICSB474 × ICSV101039 were 32.6% compared to other F_3populationsandrespectiveparents.Further,theleastNDFdigestibilitywasfoundin ICSB474× ICSV101112 with 60.66%, and the highest was seen in SSV84 × ICSV101039 with 82.66%compared to its respective recipient, donor and wild type plants, IVOMD% was least inICSV15024 ×N609 with 36% and highest in ICSV15024 ×ICSV101112 with 64%. Inaddition, the lowest hemicellulose percentage was observed in ICSB474×N600 at 19.29%and the highest in ICSV15024 ×N609 at 29.51%. Details of the individual BLUP's meanvaluesarementioned inTable4.

Table4-PartiallyReplicatedExperimentalDesign(BLUP'sValues)

S.No	Category F_3 , (RP & DPParents)	bmr/WT	Adf %	ME (MJ/ kg)	adl%	Ash %	Ndf %	ivomd %	Cellulose(%)	HemiCellulose(%)
1	ICSB38×N600	F_3*bmr*12	49.89	5.98	5.10	8.07	77.89	44	43.53	28.86
2	ICSB38 ×N600.	F_3*bmr*12	47.15	6.92	5.60	10.86	69.73	43	40.61	23.11
3	SSV84 ×N609	F_3*bmr*6	45.39	6.83	4.99	9.38	70.06	47	39.84	25.08
4	SSV84 ×N609	F_3*bmr*6	46.07	6.95	4.87	10.17	70.81	47	40.27	24.99
5	ICSB38 XN600	F_3*bmr*12	45.56	6.72	4.18	9.77	71.10	47	40.70	27.19
6	ICSB38 XN600	F_3*bmr*12	44.85	7.19	4.91	9.94	69.56	43	39.84	25.18
7	ICSV100324× ICSV101039	F_3*bmr*12	45.40	7.30	5.04	8.36	69.58	45	39.73	25.26
8	ICSV18003×N609	F_3*bmr*6	40.84	7.77	3.91	5.99	66.49	53	37.57	26.02
9	ICSV18003×N609	F_3*bmr*6	45.26	7.54	4.85	6.61	71.05	45	40.51	26.12
10	SSV84 ×N609	F_3*bmr*6	47.55	6.77	4.55	9.90	73.38	48	43.87	24.77
11	SSV84 ×N609	F_3*bmr*6	45.19	6.88	4.71	10.80	70.99	42	41.81	23.46
12	SSV84 ×ICSV101039	F_3*bmr*12	46.82	6.63	5.10	9.24	75.52	49	41.64	27.69
13	SSV84 ×ICSV101039	F_3*bmr*12	49.51	7.06	5.02	10.77	73.09	43	44.14	22.95
14	SSV84 ×N609	F_3*bmr*6	38.07	8.19	3.91	8.87	62.68	40	34.24	23.20
15	ICSB38 ×ICSV101039	F_3*bmr*12	48.61	6.51	5.57	9.26	76.70	57	43.15	28.22
16	ICSV100324× ICSV101039	F_3*bmr*12	47.32	6.61	4.48	10.33	71.94	49	42.16	25.22
17	SSV84 ×ICSV101039	F_3*bmr*12	49.98	6.16	7.18	7.35	78.73	47	42.91	28.87
18	ICSB474×N609	F_3*bmr*6	51.62	6.30	4.85	13.11	74.26	44	46.93	22.27
19	SSV84 ×ICSV101039	F_3*bmr*6	53.73	6.59	5.54	8.44	82.56	61	48.27	29.34
20	ICSV100324× ICSV101039	F_3*bmr*12	49.38	6.43	5.00	13.75	69.41	48	42.22	22.09
21	ICSV15024× ICSV101112	F_3*bmr*6	48.83	6.97	5.48	10.66	74.58	38	42.79	25.38
22	ICSV15024 × ICSV101112	F_3*bmr*6	48.94	5.74	5.97	6.26	77.17	64	43.74	29.23
23	ICSB474×N609	F_3*bmr*6	43.28	8.01	4.04	9.11	63.71	46	35.42	23.18
24	SSV84 ×ICSV101039	F_3*bmr*12	59.69	6.69	4.08	11.75	82.66	44	51.52	23.11
25	ICSB38 ×ICSV101039	F_3*bmr*12	45.23	7.21	4.89	8.39	70.82	49	40.26	26.39

26	ICSB474×ICSV101112	F_3*bmr*6	42.18	7.86	4.54	5.68	70.43	44	38.13	25.98
27	ICSB474×ICSV101112	F_3*bmr*6	42.18	6.93	4.47	13.09	60.66	52	37.07	21.57
28	ICSB38×ICSV101039	F_3*bmr*12	50.08	6.44	4.23	11.12	72.90	58	44.16	24.28
29	SSV84×ICSV101112	F_3*bmr*6	51.23	6.30	5.51	9.11	76.69	41	44.70	25.34
30	SSV84×ICSV101112	F_3*bmr*6	49.96	6.17	4.79	10.71	76.07	52	45.58	25.33
31	ICSV100324× ICSV101039	F_3*bmr*12	42.85	6.84	4.07	8.00	66.44	52	37.10	25.52
32	SSV84 ×N609	F_3*bmr*6	34.59	8.15	4.84	3.73	64.63	46	32.59	27.29
33	ICSB474×ICSV101112	F_3*bmr*6	45.03	6.80	4.53	7.97	69.01	45	40.01	25.12
34	ICSB474×N600	F_3*bmr*12	45.54	7.06	4.94	5.15	73.10	43	39.53	26.31
35	ICSV15024×N609	F_3*bmr*6	50.47	6.37	4.66	8.43	74.67	44	44.08	25.77
36	ICSV15024×N609	F_3*bmr*6	44.27	7.01	4.39	7.89	76.75	36	41.60	29.51
37	ICSB474×ICSV101039	F_3*bmr*12	43.05	7.58	4.61	8.84	68.30	43	39.46	23.72
38	ICSB474×ICSV101039	F_3*bmr*12	41.86	7.58	5.03	4.56	62.67	41	32.83	24.46
39	ICSB474×ICSV101112	F_3*bmr*6	54.32	6.30	4.22	7.85	79.35	48	48.14	24.61
40	ICSB474×ICSV101039	F_3*bmr*12	46.46	6.76	4.27	11.85	68.84	50	41.05	22.71
41	ICSB474×ICSV101112	F_3*bmr*6	43.21	6.88	4.26	10.84	67.53	45	39.50	24.71
42	ICSB474×N600	F_3*bmr*12	53.68	6.18	4.98	11.39	75.37	43	47.92	19.29
43	SSV84×ICSV101039	F_3*bmr*12	45.07	6.77	5.41	9.39	71.40	51	39.13	26.30
44	SSV84×ICSV101039	F_3*bmr*12	51.11	6.34	5.49	10.12	79.06	37	48.16	24.48
45	ICSB474×ICSV101039	F_3*bmr*12	39.36	8.18	4.02	6.51	63.29	42	32.60	25.76
46	ICSB474×ICSV101112	F_3*bmr*6	47.35	6.78	5.01	9.17	67.29	46	41.07	22.02
47	ICSV18003(RP)	WT	37.04	8.56	5.84	5.11	61.68	45	33.95	24.08
48	ICSV100324(RP)	WT	42.24	7.68	5.08	7.19	66.90	45	37.28	24.03
49	SSV84(RP)	WT	45.68	6.79	5.33	8.30	72.39	48	40.60	26.49
50	ICSV15024(RP)	WT	40.95	7.54	5.92	4.08	67.21	50	35.92	26.01
51	ICSV101039(DP)	*bmr*12	46.31	6.70	5.64	10.74	70.41	48	41.57	24.47
52	N609(DP)	*bmr*6	42.86	7.18	5.60	11.83	66.50	48	37.82	23

ADF-Acid Detergent Fibre, ADL- Acid Detergent Lignin, NDF-Neutral Detergent Fibre,IVOMD-In-Vitro Organic Matter Digestibility, ME- Metabolizable Energy, DM-Dry Matter,RP-RecurrentParent,WT-WildType,DP-DonorParent**,**BLUPs-BestLinearUnbiasedPredictions.

Discussion

In this study, the lignin concentration was found to be the least in *bmr6* lines of *the* F_3*population* (ICSV18003 ×N609, SSV84 ×N609), which was in agreement with the studiesdone by other researchers (Barrière *et al.,* 2007 and Ceballos *et al.* 2009). Our study outcomes are based on earlier research findings, which can be attributed to the combinedinfluence of the sweet stem and *BMR* traits. Research by Oliveira *et al.* (2020) and Espinal(2009)indicatesthatgenotypeswithboththesweetstemtraitandthe*bmr*mutation(SS/HBM genotypes)synergisticallywork to decreaselignincontent in thestover.

The highest and lowest ash percentages were observed in *bmr* traits ICSB474 × N609 andSSV84 × N609, respectively, compared to WTpopulations. The natural alkalinity ofbiomassashreducesthefusionpointofashes,resultinginfouling,slagging,andcompromisedperfo rmance of combustion systems (Carrillo *et al.,* 2014). On the other hand, alkaline ashcontaining phosphorus, potassium, calcium, boron, and other mineral elements *bmr6* and*bmr12* sorghum ash by-products can be used to grow plants healthily. In the current work,however,theashpercentagewasatmost13.75%.The*bmr*linesmeetthebioethanolproductionr equirement of having an ash content of 10% or less. Exceeding this limit, leading to higherash concentrations, is undesirable as it could hinder the fermentation process and cause toolcrustduringdistillation.(Carrillo*etal.*,2014).Therefore,itcanbeinferred thatthe *bmr6*and*bmr12*mutationscan safelybedeployed in sorghum breedingforbioenergyproduction.Interestingly,animprovedcellulosecontentrelativetoWTgenot ypeswasobservedin*bmr12*,which can be considered in sorghum breeding to boost 2G bioethanol bioconversion(Habyarimana*et al.,* 2016; Tse *et al.,* 2021).

Conclusion

The *bmr* traits differed in the proximate fibre component analysis compared to WTlines. Considering the reduced lignin and ash percentage in *bmr* lines meeting the bioethanolproductionrequirement,the*bmr6*and*bmr12*genescansafely be deployedinsorghumbreedingforbioenergyproduction.

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