



Development of multinutrient-rich biofortified sweet corn hybrids through genomics-assisted selection of *shrunk2*, *opaque2*, *lcyE* and *crtRB1* genes

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Abstract

Sweet corn has gained worldwide popularity. Traditional sweet corn possesses low concentration of essential nutrients such as lysine (0.15–0.25%), tryptophan (0.03–0.04%) and provitamin-A (proA 3–4 ppm), and deficiency leads to serious health problems in humans. Here, stacking of *shrunk2* (*sh2*), *opaque2* (*o2*), *lycopene epsilon cyclase* (*lcyE*) and β -carotene hydroxylase (*crtRB1*) genes were undertaken in the parents of four hybrids viz., APQH1, APHQ4, APHQ5 and APHQ7 using marker-assisted backcross breeding (MABB). Gene-linked markers (*umc2276* and *umc1320*) for *sh2*, while gene-based markers for *o2* (*umc1066* and *phi057*), *lcyE* (*5'TE-InDel*) and *crtRB1* (*3'TE-InDel*), were used for genotyping in BC₁F₁, BC₂F₁ and BC₂F₂. Selected backcross progenies showed high recovery of recurrent parent genome (92.4–97.7%). The reconstituted sweet corn hybrids possessed significantly high lysine (0.390%), tryptophan (0.082%) and proA (21.14 ppm), coupled with high kernel sweetness (brix 18.96%). The improved sweet corn hybrids had high cob yield (12.22–15.33 t/ha) across three environments. These newly developed biofortified sweet corn hybrids possess great significance in providing balanced nutrition. This is the first report of combining *sh2*, *o2*, *lcyE* and *crtRB1* genes for enrichment of sweet corn hybrids with multiple essential nutrients.

Keywords QPM · Provitamin-A · Marker-assisted selection · Backcross · Malnutrition

Introduction

Sweet corn possesses economic importance due to its increased worldwide usage as fresh and processed vegetables (Chhabra et al. 2019). Global import of frozen sweet corn was US\$433 million, while it was US\$957 million for processed sweet corn (FAOSTAT 2017). Due to increasing

urbanization, the demand of sweet corn has increased tremendously (Lertrat and Pulam 2007; Hossain et al. 2015). It is usually consumed at immature stages of endosperm development at 20–24 days after pollination (DAP) (Khanduri et al. 2011). The green plants after harvest serve as source of green fodder to cattle.

Recessive *shrunk2* (*sh2*) has been predominantly used for sweet corn cultivars development. *Sh2* located on chromosome 3L possess six times more sucrose and reducing sugar than normal maize. The depletion of sugar level in *sh2* type is much slower and thus has extended shelf life (Lertrat and Pulam 2007). Due to this reason, *sh2*-based sweet corn is now more popular worldwide (Hossain et al. 2015). Micronutrient malnutrition is a serious health problem, and nearly two billion people suffer from deficiency of micronutrients (Global Nutrition Report 2018). Deficiency of provitamin-A (proA) leads to measles, diarrhoea, respiratory diseases, night blindness and low resistance to infectious diseases (Bouis and Saltzman 2017). The lack of adequate lysine and tryptophan affects protein biosynthesis inside the

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body and also leads to fatigue, dizziness, nausea, anaemia, delayed growth, depression, anxiety, loss of appetite and reproductive tissue (Hossain et al. 2018). Therefore, development of nutrition-rich staple crops through plant breeding approach hold great promise to address the problem of malnutrition (Pfeiffer and McClafferty 2007).

Traditional sweet corn possesses low level of lysine (0.15–0.25%), tryptophan (0.03–0.04%) and proA (3–4 ppm). The recessive *opaque2* (*o2i* enhances lysine and tryptophan by 2-fold (Mertz et al. 1964). The mutant version of β -carotene hydroxylase (*crtRB1*) and lycopene epsilon cyclase (*lcyE*) increases kernel proA by ~10-fold (Harjes et al. 2008; Yan et al. 2010). The *o2*, *crtRB1* and *lcyE* genes have been utilized for development of proA rich field corn cultivars worldwide (Prasanna et al. 2020). So far, no sweet corn hybrid enriched with lysine, tryptophan and proA has been commercialized elsewhere in the world. The present investigation was therefore undertaken to (i) combine *sh2*, *o2*, *crtRB1* and *lcyE* genes into the parents of elite hybrids using marker-assisted selection (MAS); (ii) evaluate the MAS-derived sweet corn inbreds and hybrids for kernel sweetness, lysine, tryptophan and proA; and (iii) compare the MAS-derived genotypes for agronomic and yield performance with the original versions.

Materials and methods

Plant material

Parental lines (PMI-PV5, PMI-PV6, PMI-PV7 and PMI-PV8) of four proA rich quality protein maize (QPM) hybrids, viz., APQH-1, APQH-4, APQH-5 and APQH-7, having recessive alleles of *o2*, *crtRB1* and *lcyE* developed at ICAR-Indian Agricultural Research Institute (IARI), New Delhi (Zunjare et al. 2018) were targeted for introgression of *sh2* allele using marker-assisted backcross breeding (MABB). APQH-1, APQH-4, APQH-5 and APQH-7 are the proA rich version of the released medium-late maturing QPM hybrids (HQPM-1, HQPM-4, HQPM-5 and HQPM-7) in India (Table S1). SWT-147, a sweet corn inbred with recessive *sh2* allele, was used as donor line. The pedigree details of the recurrent and donor parents are presented in Table S2.

Development of backcross progenies

Backcross- and self-populations segregating for the alleles of *sh2*, *o2*, *crtRB1* and *lcyE* were grown at two locations alternatively viz., Winter Nursery Centre (WNC), ICAR-Indian Institute of Maize Research (IIMR), Hyderabad (17° 21' 50.39" N and 78° 29' 42.31" E) and ICAR-IARI, New Delhi (29° 41' 52.13" N and 77° 0' 24.95" E). The detailed MABB scheme followed in the present study is shown in Fig. 1.

Genotyping in segregating generations

Genomic DNA was isolated from the young leaves using the modified standard cetyl trimethyl ammonium bromide (CTAB) procedure (Saghai-Maroo et al. 1984). Foreground selection was carried out in BC₁F₁, BC₂F₁ and BC₂F₂ generations using markers associated with the *sh2*, *o2*, *lcyE* and *crtRB1* genes (Table 1). Gene-linked simple sequence repeat (SSR) markers *umc1320* and *umc2276* were used for *sh2* (Hossain et al. 2015). For other three target genes, gene-based markers were used for genotyping backcross populations, viz., *umc1066* and *phi057* for *o2* (Hossain et al. 2018), *5'TE-InDel* marker for *lcyE* (Harjes et al. 2008) and *3'TE-InDel*-based marker for *crtRB1* (Yan et al. 2010). The polymerase chain reaction (PCR) protocols for all the four genes viz., *sh2* (Hossain et al. 2015), *o2* (Hossain et al. 2018), *lcyE* and *crtRB1* (Zunjare et al. 2018) standardized at Maize Genetics Unit, ICAR-IARI, New Delhi, were further modified for reducing the time duration. The amplified product was resolved on 4% Seakem LE agarose gel in case of *umc1320*, *umc2276*, *umc1066* and *phi057* and 1.5% Seakem LE agarose gel in case of *crtRB1* and *lcyE* genes. The genome-wide SSRs with near uniform coverage across 10 chromosomes of maize were employed for recovering the recurrent parent genome (RPG) in foreground positive plants of BC₁F₁, BC₂F₁ and BC₂F₂ generations. The protocol for amplification of SSRs was as per Hossain et al. (2018).

Evaluation of introgressed progenies and reconstituted hybrids

The selected BC₂F₄ progenies along with the original inbreds were evaluated in random complete block design (RCBD) with two replications during rainy season (2018) at ICAR-IARI, New Delhi. Twelve reconstituted hybrids (-A, -B and -C versions in each of the four hybrids) along with the four original versions and national check hybrid (Pusa Vivek QPM9 Improved) were evaluated in RCBD with two replications in three dates of sowing (S-I: 8th August, S-II: 28th August and S-III: 17th September) during rainy season at ICAR-IARI, New Delhi (Table 2). Selfed seeds were used for estimation of kernel sweetness (brix), lysine, tryptophan and proA. Plant height, ear height, cob length, cob diameter, number of rows per cob, number of kernels per row and male- and female-flowering were also recorded. The dehusked cob yield and green fodder weight were recorded for hybrids. Newly derived inbreds and hybrids were also characterized for 30 distinctness, uniformity and stability (DUS) characters (PPVFRA 2007).

Estimation of kernel sugar

In each entry, three plants were selfed to avoid any xenia effect. Selfed kernels (60–90 grains per genotype)

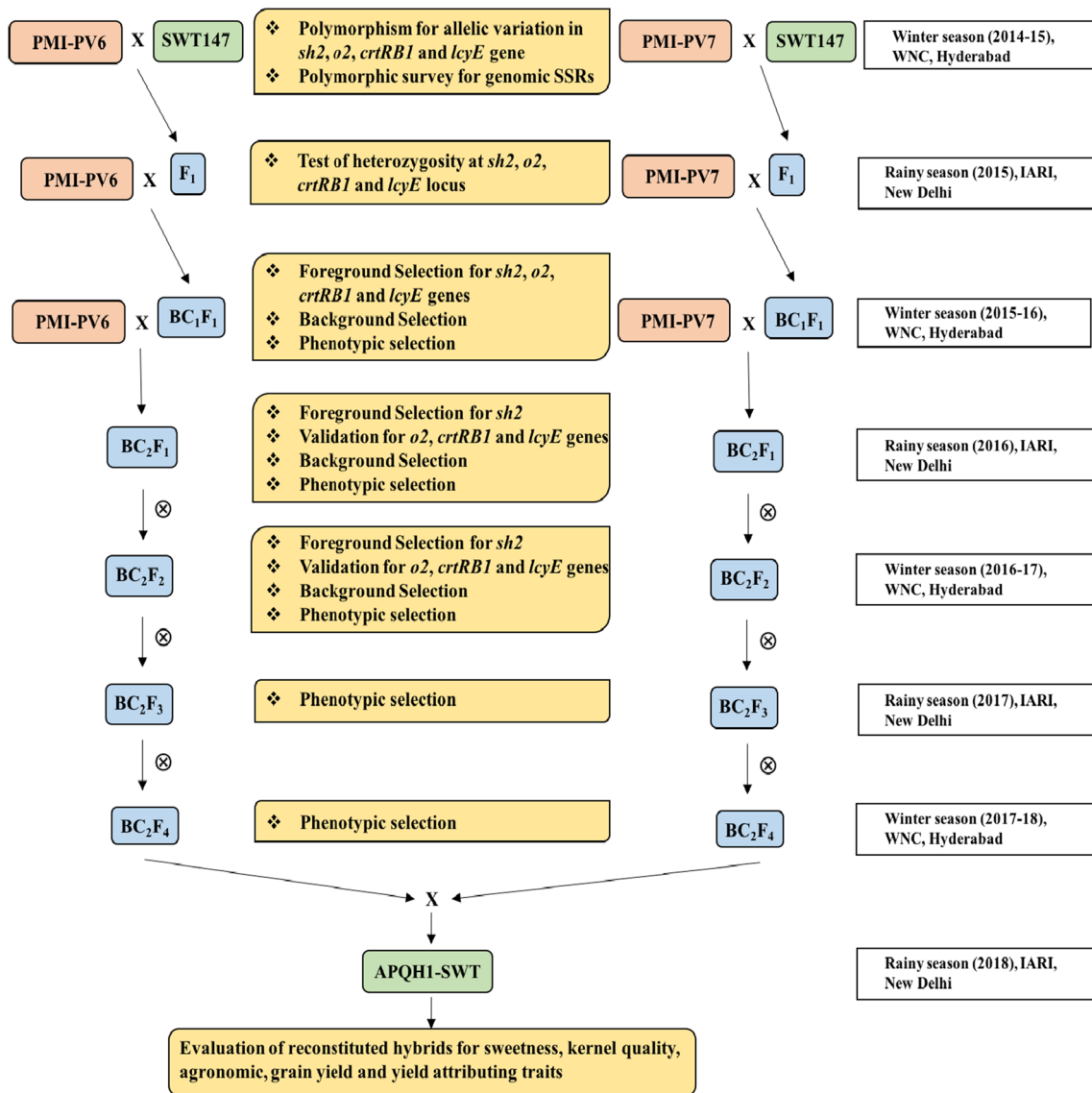


Fig. 1 Marker-assisted backcross breeding scheme (WNC, Winter Nursery Centre; IARI, Indian Agricultural Research Institute)

bulk from the three ears (20–30 grains from each ear) were crushed to measure sweetness (brix) at 20 days after pollination (DAP) using brix meter (Atago, Japan) (Khanduri et al. 2011). The grains were crushed and the milky juice thus produced was used to measure the brix reading.

Estimation of lysine, tryptophan and proA

Remaining selfed seeds from the three self-pollinated ears left after the estimation of brix at 20-DAP were stored at –80 °C until used for biochemical estimation of lysine, tryptophan and proA. A total of 20–30 selfed seeds from each of the three ears (60–90 grains in total per genotype) were dried by wrapping the seeds in Whatman® blotting paper and incubating in

desiccator filled with activated silica gel for 3 days. Deactivated silica gel was removed after 24 h with replacement of fresh activated silica gel for rapid drying. After drying, seeds were ground to fine powder to use them further for estimation of lysine and tryptophan (Hossain et al. 2018), β-carotene and β-cryptoxanthin through UHPLC System (ultra high-performance liquid chromatography; Thermo Scientific, MA, USA) (Muthusamy et al. 2014).

Statistical analysis

The observed segregation pattern of the genes was tested for goodness of fit by χ^2 analysis. The recovery of RPG was estimated as per Benchimol et al. (2005). Pooled analysis of variance (ANOVA) with fixed model was carried out using

Table 1 Details of molecular markers used for genotyping

S. No.	Markers	Gene	Bin location	Primer sequence	Reference
1.	<i>umc1320</i>	<i>sh2</i>	3.08	F-5' TGCGAAATCTGTATACCATAGGCA 3' R-5' CTCTTTTAGCAGTGTGCCGAATT 3'	Hossain et al. (2015)
2.	<i>umc2276</i>		3.08	F-5' CTAGGTAGCCAGCTAGGTACGGGT 3' R-5' AGTGGAGCTTCTCATCCTACCG 3'	
3.	<i>umc1066</i>	<i>o2</i>	7.01	F-5' ATGGAGCACGTCATCTCAATGG 3' R-5' AGCAGCAGCAACGTCTATGACACT 3'	Yang et al. (2004)
4.	<i>phi057</i>		7.01	F-5' CTCATCAGTGCCGTCGTCCAT 3' R-5' CAGTCGCAAGAAACCGTTGCC 3'	
5.	<i>5'TE-InDel</i>	<i>lcyE</i>	8.05	F-5' AAGCAGGGAAGACATTCCAG 3' R-5' GAGAGGGAGACGACGAGACAC 3'	Harjes et al. (2008)
6.	<i>3'TE-InDel</i>	<i>crtRB1</i>	10.05	F-5' ACACCACATGGACAAGTTCG 3' R1-5' AACTCTGGCCCATGAACAC 3' R2-5' ACAGCAATACAGGGGACCAG 3'	Yan et al. (2010)

F forward, R reverse

Windostat 10.0 software package. The percentage contribution of sowing dates and genotype \times sowing date for each of the traits was calculated as proportion of sum of square over total sum of square expressed in percentage.

Results

Parental polymorphism

All the four recurrent parents *viz.*, PMI-PV5, PMI-PV6, PMI-PV7 and PMI-PV8 possessed favourable allele for *o2* (150 bp

for *umc1066* and 170 bp for *phi057*), *lcyE* (650 bp) and *crtRB1* (543 bp) genes, while the donor possessed favourable allele for *sh2* gene (103 bp for *umc1320* and 130 bp for *umc2276*) and unfavourable allele of *o2* (160 bp each for *umc1066* and *phi057*), *lcyE* (300 bp) and *crtRB1* (296 bp) (Fig. 2). For *o2*, *umc1066* was used for PMI-PV5 and PMI-PV6, while *phi057* was used for PMI-PV7 and PMI-PV8. For *sh2* gene, *umc1320* was used in PMI-PV5, while *umc2276* in PMI-PV6, PMI-PV7 and PMI-PV8 for genotyping. A set of 258 genome-wide SSRs covering 10 chromosomes was screened between recurrent and donor parents (Table 3). The selected polymorphic SSRs (97–185) were employed for

Table 2 Details of check, original and reconstituted hybrids developed through crossing of specific inbreds

S. No.	Hybrids	Parents	Type
1.	Pusa Vivek QPM9 Improved	PMI-PV1 \times PMI-PV2	Check
2.	APQH-1	PMI-PV7 \times PMI-PV6	Original hybrid
3.	APQH-1-SWT-A	PMI-PV7-66-67-24 \times PMI-PV6-44-63-32	Reconstituted hybrid
4.	APQH-1-SWT-B	PMI-PV7-69-21-4 \times PMI-PV6-1-30-21	Reconstituted hybrid
5.	APQH-1-SWT-C	PMI-PV7-66-7-15 \times PMI-PV6-1-30-21	Reconstituted hybrid
6.	APQH-4	PMI-PV8 \times PMI-PV5	Original hybrid
7.	APQH-4-SWT-A	PMI-PV8-51-93-18 \times PMI-PV5-70-16-8	Reconstituted hybrid
8.	APQH-4-SWT-B	PMI-PV8-51-71-17 \times PMI-PV5-70-16-8	Reconstituted hybrid
9.	APQH-4-SWT-C	PMI-PV8-51-29-11 \times PMI-PV5-70-79-22	Reconstituted hybrid
10.	APQH-5	PMI-PV6 \times PMI-PV5	Original hybrid
11.	APQH-5-SWT-A	PMI-PV6-1-32-22 \times PMI-PV5-70-79-22	Reconstituted hybrid
12.	APQH-5-SWT-B	PMI-PV6-1-30-21 \times PMI-PV5-70-16-8	Reconstituted hybrid
13.	APQH-5-SWT-C	PMI-PV6-44-63-32 \times PMI-PV5-70-16-8	Reconstituted hybrid
14.	APQH-7	PMI-PV7 \times PMI-PV5	Original hybrid
15.	APQH-7-SWT-A	PMI-PV7-66-7-15 \times PMI-PV5-70-79-22	Reconstituted hybrid
16.	APQH-7-SWT-B	PMI-PV7-66-67-24 \times PMI-PV5-70-16-8	Reconstituted hybrid
17.	APQH-7-SWT-C	PMI-PV7-66-67-24 \times PMI-PV5-70-95-13	Reconstituted hybrid

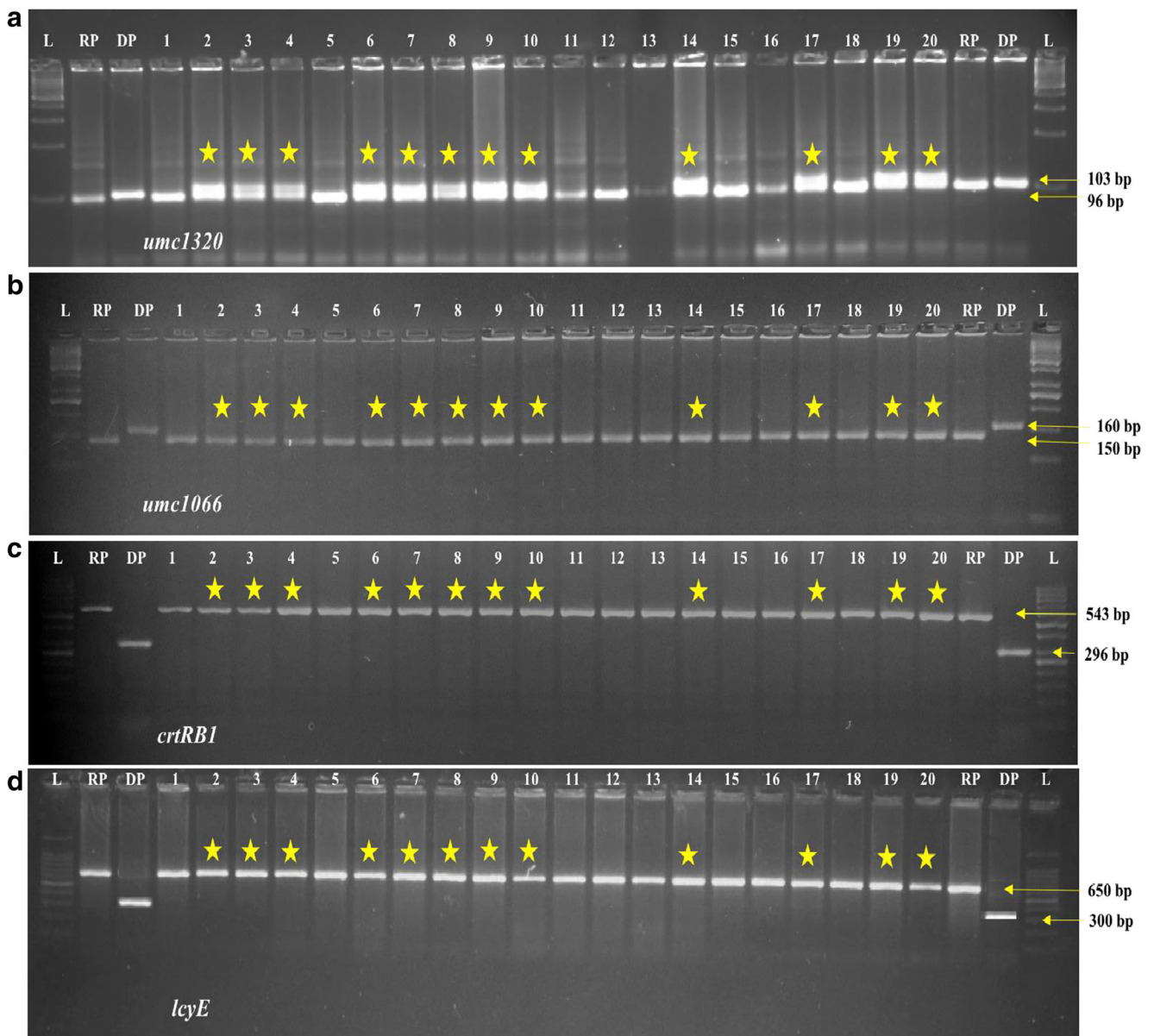


Fig. 2 Segregation of *sh2* (a), *o2* (b), *crtRB1* (c) and *lcyE* (d) genes in BC₂F₁ generation. (Star indicates desirable genotypes.) RP, recurrent parent; DP, donor parent

background selection in BC₁F₁, BC₂F₁ and BC₂F₂ generations for recovery of RPG.

Genotyping of backcross progenies

In BC₁F₁ generation, 112–189 plants were genotyped for *sh2*, *o2*, *lcyE* and *crtRB1* (Tables 4 and 5). In the case of PMI-PV5- and PMI-PV7-based progenies, *o2* showed segregation distortion (SD), while PMI-PV8 progenies deviated from expected ratio for *sh2*, *lcyE* and *crtRB1*. The RPG among selected seven progenies (heterozygous: *sh2*, homozygous: *o2*, *lcyE* and *crtRB1*) ranged from 73.5 to 85.3% (Table S3). In BC₂F₁, 93–146 plants across populations were genotyped. *Sh2*

followed 1:1 segregation in all populations, barring PMI-PV8-based one (Table 4). The RPG among 12 selected progenies (heterozygous: *sh2*, homozygous: *o2*, *lcyE* and *crtRB1*) varied from 87.1 to 96.1%. Among BC₂F₂ seeds (borne on BC₂F₁ cob), *sh2* gene segregated with a ratio of 3 (round): 1 (shrunken) in all the four populations (Table 4). In total, 222, 180, 138 and 140 phenotypically shrunken (homozygous for *sh2*) seeds were identified from the selected cobs of PMI-PV5-, PMI-PV6-, PMI-PV7- and PMI-PV8-based populations, respectively. A total of 95–150 plants in BC₂F₂ were genotyped, and progenies with homozygosity at *sh2*, *o2*, *lcyE* and *crtRB1* were selected. The percentage of RPG among selected progenies ranged 92.4–97.7%.

Table 3 Number of screened SSRs used for background selection and its percent polymorphism

Chromosome	Number of SSRs screened	PMI-PV5		PMI-PV6		PMI-PV7		PMI-PV8	
		NP	Polymorphism (%)	NP	Polymorphism (%)	NP	Polymorphism (%)	NP	Polymorphism (%)
1.	34	16	47.05	14	41.18	15	44.12	23	67.65
2.	26	16	61.54	10	38.46	17	65.38	17	65.38
3.	20	14	70.00	8	40.00	13	65.00	14	70.00
4.	25	14	56.00	9	36.00	16	64.00	18	72.00
5.	24	15	62.50	13	54.17	14	58.33	15	62.50
6.	21	11	52.38	8	38.09	15	71.43	14	66.67
7.	33	25	75.76	10	30.30	19	57.56	28	84.85
8.	24	21	87.50	9	37.50	19	79.17	17	70.83
9.	29	17	58.62	7	24.18	14	48.28	20	68.97
10.	22	12	54.54	9	40.91	13	59.09	19	86.36
Total	258	161	62.40	97	37.60	155	60.08	185	71.71

NP number of polymorphic SSRs

Nutritional quality among introgressed progenies

Kernel sweetness

Original parents, PMI-PV5, PMI-PV6, PMI-PV7 and PMI-PV8, had low brix of 8.90%, 9.25%, 8.90% and 8.50%, respectively. Among the introgressed progenies, PMI-PV7-69-21-4 possessed the highest brix (19.25%), followed by PMI-PV5-70-95-13 (18.25%), PMI-PV8-51-71-17 (17.95%) and PMI-PV6-1-32-22 (17.90%) and in each of the four genetic background (Table 6). Thus, the introgressed progenies showed 2-fold enhancement in brix compared to their original inbreds. Though introgressed progenies had higher brix, variation was observed among progenies within each pedigree lineage. For example, PMI-PV7-69-21-4 had 19.25% brix, while PMI-PV7-66-7-15 possessed 17.75% brix.

Lysine, tryptophan and provitamin-A

Original inbreds showed variation for lysine (0.312–0.344%), tryptophan (0.077–0.093%) and proA (15.89–18.52 ppm). However, PMI-PV5 had the highest lysine, tryptophan and proA. While PMI-PV8 possessed lowest lysine and tryptophan, PMI-PV6 possessed the lowest proA. MABB-derived progenies were comparable for lysine, tryptophan and proA to their respective original inbreds (Table 6). The lysine among the improved progenies of PMI-PV5 was 0.317–0.354%, while the same for PMI-PV6, PMI-PV7 and PMI-PV8 were 0.306–0.345%, 0.286–0.351% and 0.290–0.331%, respectively. Similarly, tryptophan among the progenies of PMI-PV5 (0.086–0.093%), PMI-PV6 (0.077–0.094%), PMI-PV7 (0.078–0.086%) and PMI-PV8 (0.075–0.086%) were also high. The proA among the MABB-derived introgressed

progenies of PMI-PV5, PMI-PV6, PMI-PV7 and PMI-PV8 ranged from 16.82 to 20.19 ppm, 14.87 to 18.12 ppm, 14.25 to 18.40 ppm and 15.48 to 20.06 ppm. The same in the original version was 18.52 ppm, 15.89 ppm, 16.62 ppm and 17.61 ppm, respectively (Table 6). Variation was also observed among the introgressed progenies of the same lineage. For example, PMI-PV5-70-79-22 possessed the highest lysine (0.354%), tryptophan (0.093%) and proA (20.19 ppm) compared to PMI-PV5-70-16-8 and PMI-PV5-70-95-13. Similarly, PMI-PV8-51-71-17 had the highest nutritional values over PMI-PV8-51-29-11 and PMI-PV8-51-93-18.

Agronomic performance of introgressed progenies

The introgressed progenies of PMI-PV5, PMI-PV6, PMI-PV7 and PMI-PV8 showed great degree of similarity with their original versions in relation to plant height, ear height, cob length, cob diameter, number of rows per cob and number of kernels per row. The flowering behaviour of the introgressed progenies was also similar to the original versions (Table S4). DUS characterization also showed high resemblance for majority of the characters (Table S5a and b). However, all the sweet corn progenies lack purple pigmentation in adventitious roots, stem, leaf sheath, silk and glumes. While original inbreds possessed pigmentation in some of the plant parts mentioned above.

Nutritional quality among reconstituted hybrids

Kernel sweetness

Sowing dates and genotype × sowing date had minor effects (<2% of the total variation) on brix (Table S6a). Across

Table 5 Segregation pattern of *cr1RBI* and *cyE* gene in two backcross generations

Parent	Stage	Number of plants genotyped	LL	L ⁺ L	L ⁺ L ⁺	df	χ ²	P-value	Number of plants genotyped	CC	C ⁺ C	C ⁺ C ⁺	df	χ ²	P-value
PMI-PV5	BC ₁ F ₁	116	62	54	--	1	0.551724	0.457624	116	64	52	--	1	1.241379	0.2652
	BC ₂ F ₁	93	93	--	--	--	--	--	93	93	--	--	--	--	--
	BC ₂ F ₂	150	150	--	--	--	--	--	150	150	--	--	--	--	--
PMI-PV6	BC ₁ F ₁	112	51	61	--	1	0.892857	0.34472	112	57	55	--	1	0.035714	0.8501
	BC ₂ F ₁	123	123	--	--	--	--	--	123	123	--	--	--	--	--
	BC ₂ F ₂	142	142	--	--	--	--	--	142	142	--	--	--	--	--
PMI-PV7	BC ₁ F ₁	142	63	79	--	1	1.802817	0.179374	142	82	60	--	1	3.40841	0.0649
	BC ₂ F ₁	146	146	--	--	--	--	--	146	146	--	--	--	--	--
	BC ₂ F ₂	95	95	--	--	--	--	--	95	95	--	--	--	--	--
PMI-PV8	BC ₁ F ₁	189	142	47	--	1	24.4709	<0.00001	189	76	113	--	1	7.243386	0.007117
	BC ₂ F ₁	123	123	--	--	--	--	--	123	123	--	--	--	--	--
	BC ₂ F ₂	101	101	--	--	--	--	--	101	101	--	--	--	--	--

Genes with + is wild type (unfavourable allele)

Table 4 Segregation pattern of *shrunken2* and *opaque2* gene in two backcross generations

Parent	Stage	Number of plants genotyped	Sh ⁺ Sh ⁺	Sh ⁺ Sh	ShSh	df	χ ²	P-Value	Number of plants genotyped	O2O2	O2 ⁺ O2	O2 ⁺ O2 ⁺	df	χ ²	P-value
PMI-PV5	BC ₁ F ₁	116	48	68	--	1	3.448276	0.063321	116	45	71	--	1	5.827586	0.0158
	BC ₂ F ₁	93	38	55	--	1	3.107527	0.077932	93	93	--	--	--	--	--
	BC ₂ F ₂	932*	710	222	222	--	--	--	150	150	--	--	--	--	--
PMI-PV6	BC ₁ F ₁	112	50	62	--	1	1.285714	0.256842	112	54	58	--	1	0.142857	0.7055
	BC ₂ F ₁	123	53	70	--	1	2.349593	0.125323	123	123	--	--	--	--	--
	BC ₂ F ₂	780*	600	180	180	--	--	--	142	142	--	--	--	--	--
PMI-PV7	BC ₁ F ₁	142	75	67	--	1	0.450704	0.502003	142	51	91	--	1	11.26761	0.0008
	BC ₂ F ₁	146	82	64	--	1	2.219178	0.136313	146	146	--	--	--	--	--
	BC ₂ F ₂	580*	442	138	138	--	--	--	95	95	--	--	--	--	--
PMI-PV8	BC ₁ F ₁	189	80	109	--	1	4.449735	0.034907	189	102	87	--	1	1.190476	0.2752
	BC ₂ F ₁	123	45	78	--	1	8.853659	0.002925	123	123	--	--	--	--	--
	BC ₂ F ₂	575*	435	140	140	--	--	--	101	101	--	--	--	--	--

*Segregation observed in BC₂F₂ seeds of selected BC₂F₁ cobs, genes with + is wild type (unfavourable allele)

Table 6 Nutritional quality parameters of MAS-derived inbreds

S. No.	Inbreds	Lysine (%)	Tryptophan (%)	Provitamin-A (ppm)	Brix (%)
1.	PMI-PV5	0.344	0.091	18.52	8.90
2.	PMI-PV5-70-16-8	0.334	0.086	17.26	17.75
3.	PMI-PV5-70-79-22	0.354	0.093	20.19	17.65
4.	PMI-PV5-70-95-13	0.317	0.088	16.82	18.25
5.	PMI-PV6	0.315	0.088	15.89	9.25
6.	PMI-PV6-1-30-21	0.345	0.094	14.87	17.75
7.	PMI-PV6-1-32-22	0.320	0.085	15.15	17.90
8.	PMI-PV6-44-63-32	0.306	0.077	18.12	17.30
9.	PMI-PV7	0.341	0.078	16.62	8.90
10.	PMI-PV7-69-21-4	0.328	0.082	18.40	19.25
11.	PMI-PV7-66-7-15	0.351	0.078	14.25	17.75
12.	PMI-PV7-66-67-24	0.286	0.086	16.69	17.90
13.	PMI-PV8	0.312	0.077	17.61	8.50
14.	PMI-PV8-51-29-11	0.304	0.075	15.48	17.80
15.	PMI-PV8-51-71-17	0.331	0.086	20.06	17.95
16.	PMI-PV8-51-93-18	0.290	0.081	17.65	17.75
	Standard Error	0.025	0.004	0.526	0.567

sowing dates (S-I, S-II and S-III), the mean brix value of reconstituted hybrids of APQH-1, APQH-4, APQH-5 and APQH-7 was 19.50%, 19.11%, 18.53% and 18.71%, as compared to 9.54%, 9.05%, 8.64% and 8.80% in the original hybrids, respectively, at 20 DAP (Table 7; Fig. 3a). The same in the field corn check hybrid, Pusa Vivek QPM-9 Improved was low (8.73%). Thus, the reconstituted hybrids recorded 2-fold increase in kernel sweetness due to introgression of recessive *sh2* gene. However, variation was observed among the reconstituted hybrids of same lineage. For example, APQH-1-SWT-C had the highest brix (20.24%) compared to APQH-1-SWT-B (19.39%) and APQH-1-SWT-A (18.86%).

Lysine, tryptophan and provitamin-A

Smaller variation in lysine (6.40%), tryptophan (1.22%) and proA (2.18%) was contributed by sowing dates. However, genotype × sowing date interaction had moderate influence on lysine (34.94%) and tryptophan (23.97%), while it had minor influence on proA (6.82%) (Table S6a). In each of the hybrid versions across sowing time, APQH-5-SWT-B (0.416%) had the highest accumulation of lysine followed by APQH-4-SWT-A and APQH-4-SWT-C (0.401% in each), APQH-7-SWT-A (0.393%) and APQH-1-SWT-A (0.372%). In case of tryptophan, APQH-4-SWT-B (0.092%) possessed

Table 7 Nutritional quality parameters (across three locations) of reconstituted hybrids

S. No.	Hybrids	Lysine (%)	Tryptophan (%)	Provitamin-A (ppm)	Brix (%)
1.	Pusa Vivek QPM9 Improved	0.367	0.073	19.62	8.73
2.	APQH-1	0.352	0.079	19.29	9.54
3.	APQH-1-SWT-A	0.372	0.079	19.35	18.86
4.	APQH-1-SWT-B	0.355	0.076	18.70	19.39
5.	APQH-1-SWT-C	0.358	0.077	19.28	20.24
6.	APQH-4	0.393	0.087	21.73	9.05
7.	APQH-4-SWT-A	0.401	0.091	21.59	18.90
8.	APQH-4-SWT-B	0.398	0.092	21.30	19.73
9.	APQH-4-SWT-C	0.401	0.091	21.57	18.70
10.	APQH-5	0.419	0.087	19.78	8.64
11.	APQH-5-SWT-A	0.413	0.090	20.16	18.76
12.	APQH-5-SWT-B	0.416	0.088	19.91	18.21
13.	APQH-5-SWT-C	0.415	0.082	20.40	18.61
14.	APQH-7	0.384	0.074	23.80	8.80
15.	APQH-7-SWT-A	0.393	0.072	23.76	18.76
16.	APQH-7-SWT-B	0.386	0.075	24.00	18.29
17.	APQH-7-SWT-C	0.375	0.073	23.66	19.09
	SE	0.008	0.003	0.330	0.276

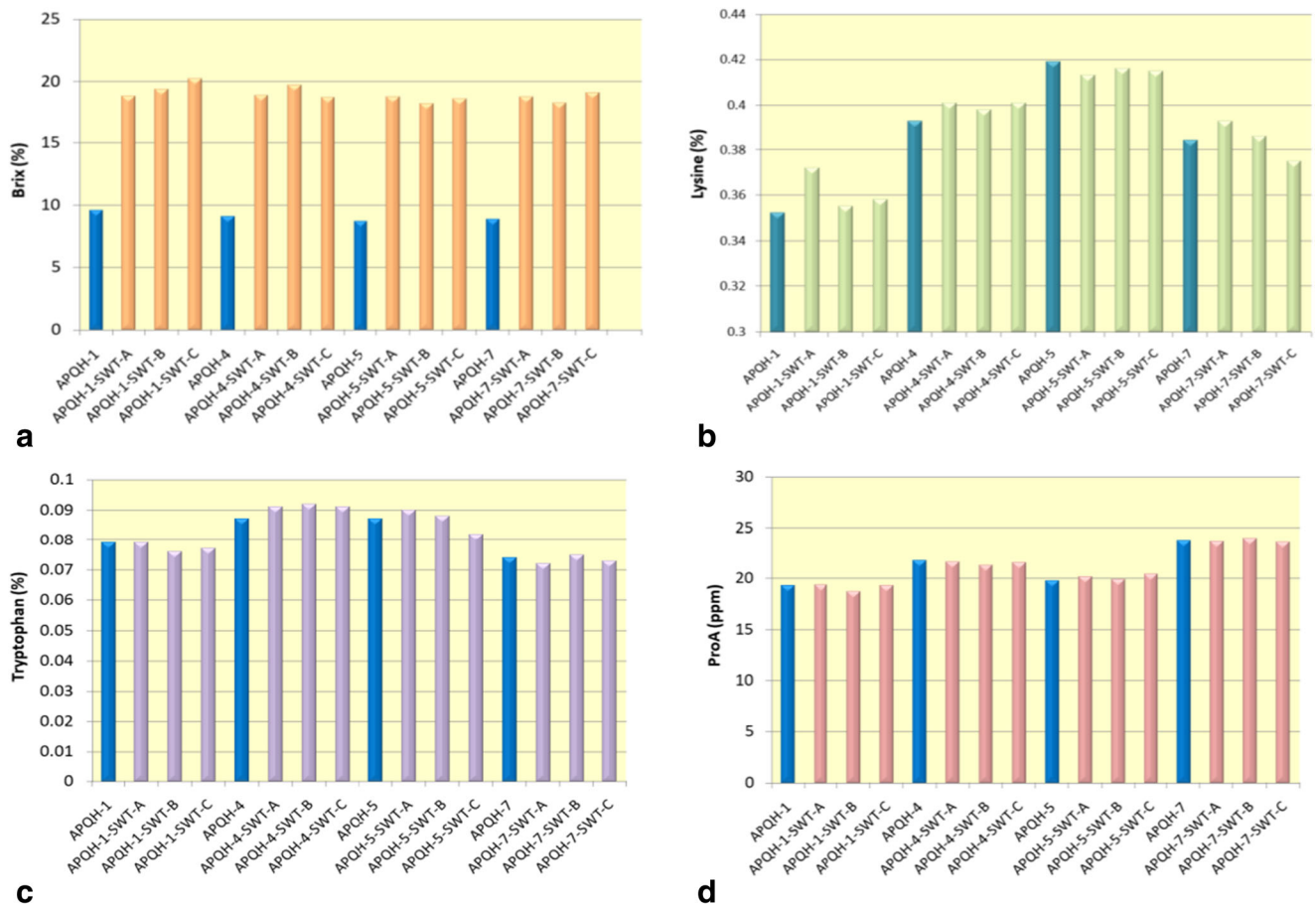


Fig. 3 a Kernel brix in original and reconstituted hybrids. b Kernel lysine in original and reconstituted hybrids. c Kernel tryptophan in original and reconstituted hybrids. d Kernel proA in original and reconstituted hybrids

the highest accumulation, followed by APQH-5-SWT-A (0.090%), APQH-1-SWT-A (0.079%) and APQH-7-SWT-B (0.075%). Among each of the hybrid versions, the highest accumulation of proA was observed in APQH-7-SWT-B (24.00 ppm), APQH-4-SWT-A (21.59 ppm), APQH-5-SWT-C (20.40 ppm) and APQH-1-SWT-A (19.35 ppm) (Table 7; Fig. 3b, c and d). Variations were also observed among reconstituted hybrids of similar lineage. For example, APQH-1-SWT-A had the highest lysine (0.372%), tryptophan (0.079%) and proA (19.35 ppm) compared to other versions (APQH-1-SWT-B and APQH-1-SWT-C).

Agronomic performance of the reconstituted hybrids

Sowing date and genotype × sowing date interaction had the minor influence on cob yield (1.50% and 16.56%) and fodder yield (3.40% and 3.59%) (Table S6b). Across dates of sowing, the reconstituted hybrids showed high degree of resemblance for various agronomic characters with their original versions (Table S7a and b). The cob yield among the original hybrids was 11.56–14.67 t/ha, while the same in the reconstituted sweet corn hybrids was 12.22–15.33 t/ha. The reconstituted hybrids also showed similar plant and cob characteristics. The DUS characterization also

showed high degree of similarity among the reconstituted and original hybrids (Table S8a and b). However, all the original hybrids had purple pigmentation in various plant parts like, glume, silk, leaf sheath, stem base and adventitious roots, while they were absent in the reconstituted versions.

Discussion

Segregation of genes and recovery of RPG

In BC₁F₁ and BC₂F₁, SD was observed for *sh2*, *o2*, *crtRB1* and *lcyE* in some of the progenies. This could be due to various gametophytic factors, defective kernel mutants, male sterility and embryo-specific mutation (Neuffer et al. 1997). Earlier, Muthusamy et al. (2014) and Zunjare et al. (2018) reported SD for *crtRB1*, while Hossain et al. (2018) observed SD for *o2*. SD thus necessitates raising of the large backcross populations in order to select desirable number of the positive segregants. Background selection was successfully used for high recovery of RPG. The average recovery of RPG after two backcrosses is 87.13%; however, the SSRs distributed throughout the genome helped achieving higher RPG. Various researchers have reported

high RPG in the two-generation based MABB programme (Muthusamy et al. 2014; Hossain et al. 2018; Zunjare et al. 2018). The high recovery also led to the high degree of resemblance with their corresponding recurrent parents with respect to plant, ear and grain characteristics.

Enhancement of kernel sweetness

The introgressed progenies and the reconstituted hybrids recorded 2-fold increase in brix. This is due to the use of closely linked markers which led to the indirect selection of the *sh2* gene (Hossain et al. 2015). The *sh2* gene, located on the chromosome 3, codes for a large subunit of ADP-glucose pyrophosphorylase (AGPase) and is involved in the synthesis of ADP-glucose from glucose-1-phosphate and adenosine triphosphate. Since the recessive version of the *sh2* blocks the conversion, starch biosynthesis such as amylose and amylopectin is restricted, leading to the higher accumulation of sugars in the kernels at 20–24 DAP (Chhabra et al. 2019). Though all the MAS-derived genotypes possessed same *sh2* allele, they reported moderate level of variation in brix (17.30–19.25%). This variation of kernel brix is attributed to various modifier loci influencing *sh2* gene (Mehta et al. 2020).

Accumulation of high lysine, tryptophan and proA

In the present study, the sweet corn hybrids possessed significantly high mean lysine (0.390%) and tryptophan (0.082%) due to the presence of recessive *o2* gene. The recessive *o2* mutant located on chromosome 7 enhances lysine and tryptophan by double (Mertz et al. 1964). The *o2* codes for basic leucine-zipper (bZIP) transcriptional factor that regulates the expression of 22-kDa α -zein genes. The enhancement is due to (i) less affinity of binding to α -zein genes, (ii) reduced transcription of lysine keto-reductase (LKR), and (iii) synthesis of various lysine-rich proteins and enzymes (Hossain et al. 2018). The high proA (21.14 ppm) observed in the sweet corn was due to *lcyE* (chromosome 8) that converts the flux of lycopene more towards β -branch, thereby leading to the formation of more β -carotene (Harjes et al. 2008). A transposable element (TE) in the 5'UTR of the *lcyE* is responsible for the polymorphism. On the other hand, *crtRB1* (chromosome 10) causes hydroxylation of β -carotene and β -cryptoxanthin into non-proA carotenoid viz., zeaxanthin. The natural variant of the *crtRB1* with no TE in the 3' UTR region drastically slows down the conversion, leading to more accumulation of proA carotenoids (Yan et al. 2010).

Though several lysine-, tryptophan- and proA-rich maize hybrids have been developed and commercialized worldwide, all these hybrids are field corn type where dried grains are used as food (Prasanna et al. 2020). However, no sweet corn hybrid with high lysine, tryptophan and proA has been developed elsewhere. In China, Yang et al. (2018) introgressed *lcyE* into four *sh2*-based sweet corn inbreds and reported low increase in proA

(2.07–3.86 ppm) over the original inbreds (0.004–3.09 ppm). However, in the present study, we have achieved very high increase in proA (>18 ppm) among the newly developed sweet corn hybrids. This high value of proA was possible due to use of *crtRB1* gene coupled with *lcyE* (Zunjare et al. 2018). However, the variation for lysine, tryptophan and proA observed among the introgressed inbreds and reconstituted hybrids is due to various modifier loci influencing *o2*, *crtRB1* and *lcyE* genes (Hossain et al. 2018, Zunjare et al. 2018). Thus, original inbreds and their improved versions were at par with lysine, tryptophan and proA due to the presence of recessive *o2* and mutant *crtRB1* genes in the recurrent parent. However, the introgressed progenies and reconstituted hybrids possessed high kernel sweetness in comparison to their original versions due to the introgression of recessive *sh2* gene. The cob and fodder yield of the reconstituted hybrids was also at par with the original versions. Thus, newly derived nutritionally rich sweet corn hybrids would provide livelihood to large number of farmers, besides providing balanced nutrition to the consumers. This is the first report of marker-assisted stacking of *sh2*, *o2*, *lcyE* and *crtRB1* genes for enhancement of essential amino acids and proA in sweet corn genotypes.

Conclusion

Traditional sweet corn lacks the desired concentration of lysine, tryptophan and proA, deficiency of which causes serious health problems. Here we combined *sh2*, *o2*, *lcyE* and *crtRB1* genes in a single genetic background using genomic-assisted breeding. The application of gene-specific molecular markers helped to precisely identify the favourable genotypes in the segregating populations. Further, stringent background selection through SSRs led to the high recovery of recurrent parent genome and similar phenotypic characters of the recurrent parents in just two backcrosses. The reconstituted hybrids possess 2-fold more lysine and tryptophan and 4-fold more proA. The molecular breeding thus helped in rapidly developing nutritious sweet corn hybrids which would have taken more than double the time in case of conventional breeding. This is the first study to combine *sh2*, *o2*, *lcyE* and *crtRB1* genes for the development of biofortified sweet corn hybrids. These multi-nutrient sweet corn hybrids would help in providing balanced food and address the malnutrition in a sustainable and cost-effective way.

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Code availability Not applicable

Author contribution Conduct of the experiments, AB; generation of backcross populations, VM and AKD; phenotypic evaluation, BKM and RUZ; statistical analysis, RUZ; biochemical analysis, AB, SJM and SS; genotyping, AB and RC; drafting of manuscript, AB, VM and FH; design of experiment, FH and KKP.

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Data availability All supporting data are included within the article and its additional files.

Declarations

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Consent to participate Not applicable

Consent for publication All the authors have read the content and consented to submit the manuscript

Competing interests The authors declare no competing interests.

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