Mol Breeding (2019) 39: 31
https://doi.org/10.1007/s11032-019-0939-x

Marker-assisted backcross breeding for enhancing β-carotene of QPM inbreds

Deepti B. Sagare · Prashant Shetti · M. Surender · S. S. Reddy

Received: 11 October 2018 / Accepted: 31 January 2019 / Published online: 12 February 2019

Abstract Maize possesses natural variations for *crtRB1* (*β-carotene hydroxylase*) and *lcyE* (*lycopene-ε-cyclase*) which significantly enhances provitamin A (proA) concentration. Enhancement of kernel proA carotenoids in QPM genetic background is of greater value. In this study, allelic variability for proA carotenoids was analyzed to identify promising β-carotene donor to introgress into QPM inbreds employing molecular breeding. Maize inbred MGU23379 (6.31 μg/g of β-carotene) was used as β-carotene donor to cross with recurrent parents (RPs), CB6-36 (CBML6) and CB7-28 (CBML7). In conversion program, F_1, BC_1F_1, BC_2F_1, BC_2F_2, BC_2F_3, and BC_2F_4 materials were generated. In each generation, foreground selection was carried out with *crtRB1*-3′TE and *umc1066*. The *crtRB1*-3′TE segregated as per the expectation. Selection for modifiers, recombinants, and background genome of RPs was carried out in BC populations. The individuals with high recovery of recurrent parent genome were retained. Tryptophan/lysine content in introgressed progenies was on par, but β-carotene content was significantly high (6.25and 6.80 μg/g) compared to original inbreds (0.71 and 1.29 μg/g). Phenotypic data recorded for different traits in the BC_2F_3 populations did not show any significant difference between the converted BC_2F_3 families and their RPs. Also, grain yield of converted inbreds (PVCBML6 and PVCBML7) was on par with their original lines. In PVCBML6, stem anthocyanin pigmentation was reduced and silk color was changed to dark pink; whereas in PVCBML7, tassel was more erect and sparer; and silk color was changed to light pink compared to original inbreds. Converted inbreds provides an ideal platform for stacking number of nutritionally important traits.

Keywords QPM · *opqaue2* · *crtRB1*-3′TE · β-Carotene · Provitamin A · Recurrent parent genome · Molecular breeding

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11032-019-0939-x) contains supplementary material, which is available to authorized users.

D. B. Sagare (✉) · P. Shetti · M. Surender · S. S. Reddy
Department of Molecular Biology and Biotechnology, PJTSAU, Hyderabad, India
e-mail: deepti.sagare@gmail.com

Introduction

Micronutrient malnutrition, caused by inadequate consumption of iron, zinc, and vitamin A, compromises the health of many populations (Kennedy et al. 2003). Worldwide, two billion people suffer from micronutrient deficiencies and about 815 million people are undernourished (Global Nutrition Report 2017: https://www.globalnutritionreport.org). Vitamin A deficiency (VAD) accounts for about 70% of the childhood deaths globally by affecting over 250 million pre-school children (Muthusamy et al. 2015). Also, protein energy malnutrition (PEM) due to inadequate proteins in diet affects billions of people worldwide (Bain et al. 2013). Deficiency of the essential amino acids, lysine and

tryptophan, causes fatigue, delayed growth, loss of appetite, depression, and anxiety in children (Nuss and Tanumihardjo 2010; Jompuk et al. 2011). Quality protein maize (QPM) is a significant promise for solving problem of PEM across the world (Nyakurwa et al. 2017), as it contains higher tryptophan and lysine in endosperm. Although cereals are rich source of energy, they are ill balanced or possesses inadequate amount of micronutrients (Nuss and Tanumihardjo 2010). To combat dietary deficiencies of protein, vitamins, and essential micronutrients, different strategies, such as industrial food-fortification, supplementation, and dietary diversification, have been tried worldwide; but none of these approaches has been found viable in the long run (Tanumihardjo et al. 2007). The development of micronutrient-enriched staple plant foods through breeding approaches, i.e., "biofortification," is a food-based solutions to combat micronutrient deficiencies (Gupta et al. 2015; Neeraja et al. 2017).

Maize is one of the most important food crops in the world, and together with rice and wheat it provides 30% of the food calories to more than 4.5 billion people in 94 developing countries (Babu and Prasanna 2014). Maize has attained importance in world economy. Maize kernels lack the essential amino acids lysine and tryptophan and are also deficient in vitamin A, C, B, iron, zinc, and iodine. Among the cereals, only the yellow kernel maize exhibits tremendous natural variation for provitamin A (proA) carotenoids to exploit through plant breeding, and being a carotenogenic plant (Rodriguez-Amaya 2001), it is considered as one of the potential model cereal crop, showing promise for proA biofortification (Zhang et al. 2012; Sagare et al. 2018). Naturally existing mutant alleles of key genes, *lycE (lycopene-ε-cyclase)* and *crtRB1 (β-carotene hydroxylase)* of carotenoid biosynthesis pathway, significantly enhances accumulation of proA compounds in the maize endosperm (Harjes et al. 2008; Yan et al. 2010; Babu et al. 2013; Azmach et al. 2013), therefore called as favorable alleles. Also, the recessive allele of *opaque2* gene (*o2*) enhances endosperm lysine and tryptophan by two folds (Mertz et al. 1964). PCR-based co-dominant markers for *lcyE*, *crtRB1*, and *o2* have been developed for their utilization in molecular breeding.

The use of molecular markers in marker-assisted backcross breeding (MABB) helps for introgression of target genes into a recipient genetic background (Babu et al. 2005) and significantly reduces the breeding cycles required for recovery of recurrent parent genome (RPG) (Gupta et al. 2013). Also, molecular markers serves as an alternative for highly expensive HPLC (high-performance liquid chromatography) analyses required for estimation of micronutrient contents among the individuals of segregating populations in MABB (Zunjare et al. 2018). A number of inbred lines/hybrids possessing favorable allele constitution at either or both the loci of *lcyE*/*crtRB1* have been developed employing MABB (Vignesh et al. 2012; Babu et al. 2013; Azmach et al. 2013; Muthusamy et al. 2014; Liu et al. 2015; Muthusamy et al. 2015; Yang et al. 2018; Zunjare et al. 2018).

The present study was undertaken to identify promising β-carotene donor using gene-based markers, introgression of favorable allele of *crtRB1* into QPM inbreds through MABB, and evaluation of MABB-derived inbreds for nutritional and other agronomic traits.

Material and methods

Plant material

Maize germplasm lines were obtained from the Indian Institute of Maize Research (IIMR), New Delhi; Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora; Maize Research Centre (MRC); and Institute of Biotechnology (IBT), Rajendranagar, Hyderabad. QPM inbreds, five of CBML6 family, and eight of CBML7 family (CBML6 and CBML7 are QPM versions of BML6 and BML7, respectively—parental lines of maize hybrid DHM117; developed at IBT using CML181 as QPM donor) were used for identification of suitable recurrent parent (online resource 1) and seventy-two (72) maize germplasm lines (online resource 2) were used for identification of promising donor for β-carotene. The study was carried out during *kharif* 2013 to *rabi* 2015–16 at IBT and MRC, Rajendranagar, Hyderabad.

Recurrent parents and donor parent

Light box screening was carried out to check the kernel opacity. The *o2* gene–based SSR *umc1066* was used to screen QPM germplasm lines and further tryptophan/lysine content was determined. Final selection of recurrent parents (RPs) was carried out on the basis of combining ability and heterosis of the QPM inbreds of CBML6 and CBML7 families (Surender et al. 2017).

Seventy-two maize inbreds were screened with functional markers of *crtRB1*-5′TE, *crtRB1*-3′TE, and *lcyE*-5′TE to study allelic variations at *β-carotene hydroxylase* (*crtRB1*)-5′TE, *lycopene-ε-cyclase* (*lcyE*)-5′TE and *β-carotene hydroxylase* (*crtRB1*)-3′TE. Several studies reported that allele1 of *crtRB1*-3′TE alone can double the β-carotene concentration in maize kernel irrespective of genetic constitution of *crtRB1*-5′TE and *lcyE*; therefore, biochemical analysis for β-carotene estimation of the maize inbreds possessing favorable allele of *crtRB1*-3′TE was carried out to identify promising donor parent (DP).

Molecular analysis

DNA was extracted from young leaves of 20-day-old plants using a modified CTAB method. PCR reaction was carried out in 10 μl reactions containing, 2 μl (25 ng/μl) maize genomic DNA, 0.3 μl (2.5pmole) of forward and reverse primers each, 1 μl (2.5 mM) dNTPs, 1 μl Taq buffer containing 15 mM $MgCl_2$ (10×), 1 μl (1 U/μl) of Taq polymerase, and 4.6 μl of sterile distilled water using the Eppendorf Thermocycler. After initial denaturation for 5 min at 94 °C, each cycle comprised 1-min denaturation at 94 °C, 45-s annealing at 55°–57 °C, and 1-min extension at 72 °C with a final extension for 10 min at 72 °C at the end of 35 cycles. The PCR products were mixed with bromophenol blue gel loading dye and were resolved by electrophoresis on 3% Metaphor™ Agarose gel (containing 0.5 mg/ml ethidium bromide) and visualized on GelDoc™ XR (Bio-Rad Laboratories Inc., USA).

Breeding scheme

For MABB scheme, both RPs were crossed with DP to obtain F_1 seeds (Fig. 1). The F_1s were backcrossed with respective RP to obtain a large number of BC_1F_1 seeds. In the BC_1F_1 generation, individual plants that were heterozygous at the target loci were identified (foreground selection) reducing the population size for further screening. From the individual plants that were heterozygous for target loci, those that were homozygous for the recipient allele at one marker locus flanking the target loci, i.e. recombinants, were identified (recombinant selection). From the recombinant plants, individuals with recurrent-type allele at amino acid and endosperm modifier loci were selected (modifier selection). After modifier selection, individuals with the less number of donor-type genic SSRs were selected (background selection). In the second backcross (BC) generation, the same strategy was followed for selection of individual plants with the desired allele combination at the target loci, flanking marker, modifier loci, and RP-type genomic composition at the non-target loci; and crosses were made with the

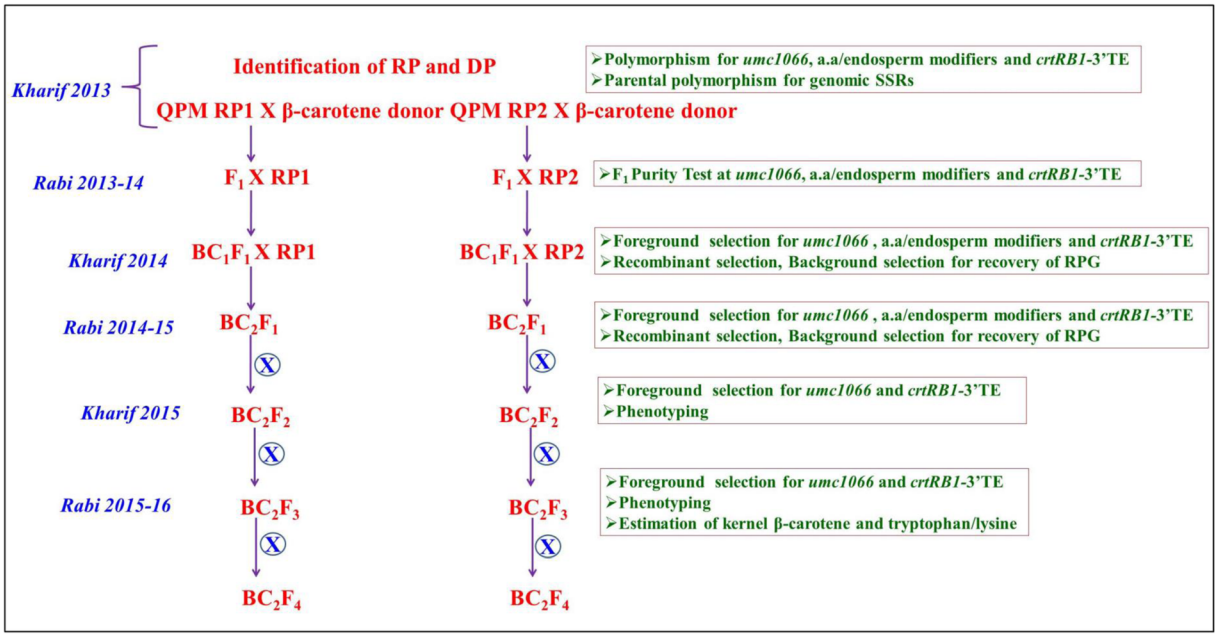

Fig. 1 MABB scheme for enrichment of β-carotene into QPM inbreds

respective RPs to obtain next generation. Selected BC_2 plants were self-pollinated in two successive generations for further analysis.

Marker assay

Functional marker of *crtRB1*-3′TE (Yan et al. 2010) and *o2* gene–specific *umc1066* marker (Babu et al. 2005) were used for F_1 confirmation and foreground (FG) selection in BC_1F_1, BC_2F_1, BC_2F_2, and BC_2F_3 generations. Flanking markers (FMs) of *crtRB1*, viz., *umc1506*, and *bnlg1028* (Chander et al. 2008), were used for recombinant (RC) selection in BC_1F_1 and BC_2F_1 generations. Six SSR markers, viz., *mmc0241*, *umc1216*, *phi072*, *bnlg1633*, *bmc1382*, and *phi075* (Danson et al. 2006) for endosperm and amino acid modifiers, were used. The sequence information of these markers (online resource 3) was taken from maize genome database (http://www.maizegdb.org). Polymorphism between DP and two RPs was surveyed using these markers. A total of 500 genomic SSRs unlinked to *crtRB1* and *o2* covering all ten chromosomes, including the *o2* and *crtRB1* carrier chromosome 7 and 10, respectively, were screened and the markers showing polymorphism between the DP and RPs were used for background (BG) selection to recover the recipient genome (online resource 6). The sequence information of these markers was taken from maize genome database. The SSRs that revealed fixed (homozygous) alleles at non-target loci at one generation were not screened at the next BC generation. Only those markers that were not fixed for the RP allele were analyzed in the following generations.

Phenotyping and biochemical analysis

Biochemical analysis for estimation of β-carotene was carried out using water alliance HPLC System. Tryptophan content of maize kernels was estimated using the method described by Hernandez and Bates (1969) and the value of lysine was calculated by multiplying tryptophan value by four as tryptophan and lysine possess strong correlation ($r = 0.99$) in *o2*-based germplasm, with value of lysine being four times that of tryptophan (Vivek et al. 2008).

The phenotypic data was recorded in BC_2F_3-generated plants during *rabi* 2015–16. Observations on different agronomic characteristics, viz., days to 50% tasseling, days to 50% silking, days to maturity, plant height, ear height, ear girth, ear length, number of kernel rows per ear, number of kernels per row, 100 seed weight, and grain yield per plant, were recorded.

Data analysis

Scoring of marker alleles was carried out for BG selection. Heterozygous alleles were represented by *H* and scored as 0.5. Homozygous recipient allele was represented by *A* and scored as 1 while homozygous donor allele was represented by letter *B* and scored as 0. Based on the scoring data, RPG was calculated using the formula given by Kumar et al. (2012).

$$\mathrm{RPG} = \frac{[A + 1/2H]}{N} x100$$

(N, total number of parental polymorphic markers screened)

For comparing agronomic traits, tryptophan/lysine content and β-carotene content between individuals of the BC_2F_3 families and the corresponding RP line statistical analysis were performed using Windostat 8.0.

Results

Allelic variation at *crtRB1*-3′TE, *lcyE*-5′TE, and *crtRB1*-5′TE and identification of promising DP

Among all the 72 inbreds screened for allelic variability study; four inbreds, viz., MGU23379, MGU23207, CM150, and BAJIM12-11, were found to contain favorable allele, i.e., allele1 (543 bp) of *crtRB1*-3′TE gene, one inbred BML13 was found to possess favorable allele, i.e., allele4 (993 bp) of *lcyE*-5′TE, and no other inbreds were found with favorable allele of *crtRB1*-5′TE (online resource 4).

According to Azmach et al. (2013), inbred lines harboring the favorable alleles of the *crtRB1*-5′TE and 3′TE produce higher levels of proA, whereas favorable allele of *lcyE* gene has weak association with levels of proA carotenoids. Babu et al. (2013) reported that allele1 of *crtRB1*-3′TE alone is responsible to double the β-carotene concentration in maize kernel irrespective of genetic constitution of *crtRB1*-5′TE and *lcyE* gene. Marker-assisted selection (MAS) for favorable allele1 of *crtRB1* alone leads to rapid doubling of total proA concentration (Babu et al. 2013). Indian maize

germplasm for this favorable allele has been screened by many researchers (Selvi et al. 2014; Zunjare et al. 2017) to identify proA donor to be used in breeding program.

Biochemical analysis for β-carotene estimation of the four inbreds containing allele1 of *crtRB1*-3′TE was carried out using HPLC method. Among the 4 inbreds, MGU23379 was found to contain high amount of β-carotene (6.31 μg/g), followed by MGU23207 (5.25 μg/g), CM150 (3.01 μg/g), and BAJIM12-11 (3.17 μg/g). Total carotenoid content was recorded highest in CM150 (7.23 μg/g), followed by MGU23379 (9.18 μg/g), MGU23207 (4.27 μg/g), and BAJIM12-11 (3.34 μg/g), respectively. As the β-carotene content was found to be high in MGU23379, it was selected as a promising β-carotene donor in MABB program to enrich β-carotene of the QPM inbreds. The β-carotene content in maize inbred BML13 possessing allele4 (993 bp) of *lcyE*-5′TE (1.31 μg/g) was lower than four inbreds carrying allele1 of *crtRB1*-3′TE.

Identification of promising QPM inbreds for conversion into β-carotene rich lines

Seeds of five QPM inbreds of CBML6 family and eight of CBML7 family were found to possess < 25% kernel opacity in light box screening. These QPM inbreds were further subjected to genotyping with *o2*-specific SSR *umc1066* and showed amplicon size of 155 bp. Tryptophan/lysine estimation of QPM inbreds of both the families was carried out.

In CBML6 family, tryptophan and lysine content (online resource 1) was high in CB6-36 (0.89%; 3.56%) and in CBML7 family it was high in CB7-28 (0.84%; 3.36%). Also, general combining ability (GCA) and heterosis studies proved that cross combination CB6-36 × CB7-28 exhibits on par grain yield per plant when compared to check DHM117 (Surender 2015). Biochemical analysis for kernel iron (Fe) and zinc (Zn) of QPM showed that CB6-36 and CB7-28 are rich sources of Fe (42 mg/kg) and Zn (48 mg/kg), respectively (Surender 2015). The β-carotene content of CB6-36 and CB7-28 is 0.71 and 1.29 μg/g, respectively. Therefore, these lines were selected for enhancing β-carotene and named them as CBML6 and CBML7, respectively.

Parental polymorphism survey

MGU23379 (β-carotene donor–DP), CBML6, and CBML7 (RPs) were screened with *crtRB1*-3′TE (Forward, Reverse1, and Reverse2), *o2*-specific *umc1066* (Forward and Reverse), five amino acid–linked SSRs (*mmc0241*, *umc1216*, *phi072*, *bnlg1633*, *bmc1382*), one endosperm modifier SSR *phi075*, two FMs (*umc1506* and *bnlg1028*), and 500 genomic SSRs to check parental polymorphism. DP showed distinct polymorphism with both RPs at *crtRB1*-3′TE, *umc1066*, *mmc0241*, *phi075*, *umc1506*, and *bnlg1028* loci (online resource 5). The rest of the modifier loci were monomorphic between DP and both RPs, and out of 500 genomic SSRs screened, DP showed polymorphism at 161 and 82 loci with CBML7 and CBML6, respectively (online resource 6).

Generation of F_1, BC populations, genotyping, and phenotyping

During *kharif* 2013, F_1 seed materials were developed by making crosses between MGU23379 and both RPs (CBML6 and CBML7). The F_1 seeds obtained from both crosses were sown as per ear to row method during *rabi* 2013–14 to generate F_1 population. Each individual of F_1 population was subjected to FG selection with *umc1066* and *crtRB1*-3′TE. Both the F_1 populations exhibited heterozygosity for *crtRB1*-3′TE and *o2* allele. FG-selected true F_1s of both the crosses were backcrossed with respective RPs to generate BC_1F_1 seed material. BC_1F_1 population was generated during *kharif* 2014. In BC_1F_1 population, 500 plants of each cross were tagged and FG selection was performed for *o2* and *crtRB1-3′TE* loci (Fig. 2). Individuals with recessive-type allele at *o2* were subjected to screen with *crtRB1-3′TE* loci and individuals possessing heterozygosity at *crtRB1-3′TE* loci were further screened with FM *umc1506* to select single recombinants (SRCs). SRC individuals were subjected to screen with polymorphic amino acid (*mmc0241*) and endosperm (*phi075*) modifiers. As the RPs possess favorable alleles for modifiers, individuals showing RP-type alleles at both of these loci were selected (Table 1). A total of 21 CB6-BC_1F_1 and 14 CB7-BC_1F_1 individuals were subjected to BG selection with 82 and 161 polymorphic SSRs to recover maximum RPG of CB6 and CB7, respectively. In the CB6-derived BC_1 progenies, the average RPG content was 76.73%, which ranged from 60.33 to 83.00%: approximately 1.73% higher than the theoretical value (75%). Whereas in CB7-BC_1F_1 individuals, the average recovery of RPG was 74.87% which ranged from 64.25 to 83.22%: approximately 0.13% lower than the

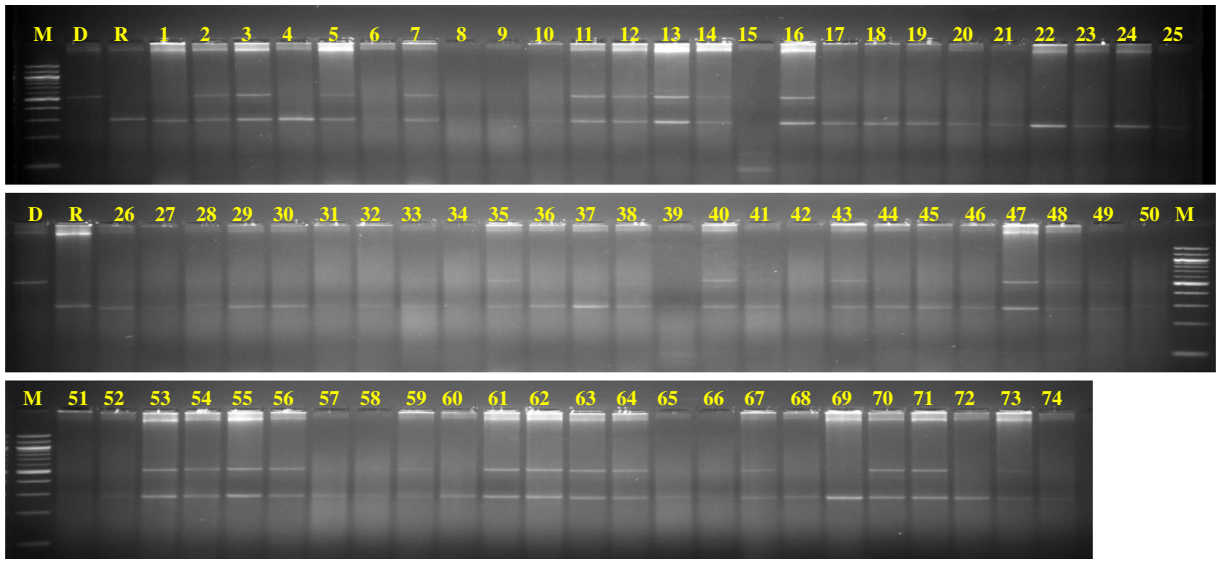

Fig. 2 Foreground selection of CBML6-BC_1F_1 with *crtRB1*-3′TE. M: 100 bp; D: MGU23379; R: CBML6; Lanes 1–74,: CBML6-BC_1F_1 population

theoretical value (75%). In CB6-BC_1F_1 and CB7-BC_1F_1 population, all the plants with the highest RPG (P_6-193: 83.00, P_6-329: 82.66, P_6-407: 82.25 and P_7-19: 83.22, P_7-487: 82.75) were backcrossed with respective RPs for generating BC_2F_1 seed material (Table 1). Chromosome-wise recovery of RPG in the best lines (CBML6: P_6-193; CBML7: P_7-19) is shown in online resource 7.

During *rabi* 2014–15, backcrossed seeds of P_6-193 and $P_7$19 individuals of CB6-BC_1F_1 and CB7-BC_1F_1, respectively, were sown to generate BC_2F_1 populations. In BC_2F_1 population (250 plants), individuals possessing recessive allele at *o2* and heterozygosity at *crtRB1-3′TE* loci were screened with FM *bnlg1028* to select double recombinants (DRCs) (online resource 8). DRCs were subjected to modifier selection and all DRCs were found to possess recurrent-type allele at modifier loci. Further, BG selection with 14 and 27 polymorphic SSRs which were not recovered in BC_1F_1 populations of CB6 and CB7, respectively, was carried out (Table 1). For CB6- and CB7-derived BC_2F_1 population, the RPG varied from 88.25 to 93.25% and 87.98 to 91.06%, with average of 90.35% and 88.09%, respectively (Table 1). The average RPG value was 2.85% and 0.59% higher than the theoretical value (87.5%) in CB6 and CB7-BC_2F_1, respectively. All the plants of CB6 and CB7-BC_2F_1 with the highest RPG (P_6-13: 91.75, P_6-178: 93.25, P_6-229: 91.34 and P_7-5: 90.25, P_7-37: 90.75, P_7-93: 89.67, P_7-112: 91.00, P_7-154: 89.20, P_7-193: 88.33, P_7-216: 91.06, P_7-238: 90.15) were selfed for generating BC_2F_2 seed material (Table 1). Chromosome-wise recovery of RPG in the best lines (CBML6: P_6-178; CBML7: P_7-216) is shown in online resource 9. The Chi square (χ^2) test for the segregation ratio of donor type and heterozygote alleles at *crtRB1-3′TE* locus was performed, which confirmed the goodness of fit of our data to the theoretical ratio of 1:1 in BC_1F_1 and BC_2F_1 generation (Table 1). BC_2F_2 seeds derived from BC_2F_1-CB6-P_6-178 and BC_2F_1-CB7-P_7-216 were used to generate BC_2F_2 population during *kharif* 2015.

During *kharif* 2015, 300 plants of each population were tagged and FG selection was performed for *crtRB1-3′TE* (Table 1). The χ^2 test for the segregation ratio of donor type, heterozygotes, and recurrent-type alleles at *crtRB1-3′TE* locus was performed, which confirmed the goodness of fit of our data to the theoretical ratio of 1:2:1 in BC_2F_2 generation (Table 1). Individuals with recurrent-type allele at *o2* loci and donor-type alleles at *crtRB1-3′TE* loci (online resource 10) were selfed to generate BC_2F_3 seed material. Harvesting was done and cobs showing similar morphology with RPs in terms of texture, size of the grains, and row arrangements were selected for recording cob phenotypic data. Further, seeds of these cobs (19 and 21 selfed cobs of CB6 and CB7-BC_2F_2, respectively) were used for estimation of β-carotene, tryptophan/lysine content. Tryptophan and lysine content varied from 0.88 to

Table 1 Molecular progress in MABB

Population	Population size	No. of plants with *opaque2* allele			No. of plants with *crtRB1-3′ TE* allele			Segregation distortion for *crtRB1-3′TE*		No. of recombinants		No. of plants with a.a./endosperm modifiers				No. of SSRs used for BG selection	No. of non-recovered BG SSRs	RPG range (%)	Mean RPG (%)	Plants with highest RPG
		D	H	R	D	H	R	χ^2	*p* value	SRC	DRC	A		B						
												H	R	H	R					
BC_1F_1-CB6	500		256	244†		119	125	0.15	0.70	64		26	38†	17	21†	82	14	60.93 to 83.00	76.73	*P_6-193 (83.00)*
																				P_6-329 (82.66)
																				P_6-407 (82.25)
BC_1F_1-CB7	500		248	252ǂ		123	129	0.14	0.70	52		28	24ǂ	10	14ǂ	161	27	64.25 to 83.22	74.87	*P_7-19 (83.22)*
																				P_7-487 (82.75)
BC_2F_1-CB6	250		4*	246ǂ		118	128	0.41	0.52		21		21ǂ		21ǂ	14	5	88.25 to 93.25	90.35	P_6-13 (91.75)
																				P_6-178 (93.25)
																				P_6-229 (91.34)
BC_2F_1-CB7	250			250		123	127	0.56	0.46		29		29ǂ		29ǂ	27	13	87.98 to 91.06	88.09	P_7-5 (90.25)
																				P_7-37 (90.75)
																				P_7-93 (89.67)
																				P_7-112 (91.00)
																				P_7-154 (89.20)
																				P_7-193 (88.33)
																				P_7-216 (91.06)
																				P_7-238 (90.15)
BC_2F_2-CB6	300			300	69	153	78	0.66	0.72											
BC_2F_2-CB7	300			300	73	148	79	0.29	0.86											
BC_2F_3-CB6	60			60	59	01†														
BC_2F_3-CB7	60			60	60															

Italicized values are denoting specific allele type which is mentioned in footnote statistical analysis has been carried out for Segregation distortion at crtRB1-3'TE with P value at 5%

*Heterozygous at *opaque2* loci—due to outcrossing with DP

ǂ Selection for RP-type allele at *opaque2, a.a, endosperm modifier loci*

† Heterozygous at *crtRB1-3′TE* loci—due to outcrossing with RP

0.90%; and 0.83 to 0.85% in CBML6- and CBML7-derived BC_2F_3 seeds, respectively (online resource 11). Tryptophan/lysine content was found to be on par with respective RPs. Content of β-carotene varied from 5.00 to 6.25 μg/g and 5.85 to 6.80 μg/g in CB6- and CB7-derived BC_2F_3 seeds, respectively (online resource 11), which is significantly high compared to RPs (Table 2). BC_2F_3-CB6 seeds derived from individuals P_6-3 and P_6-69 were found with higher β-carotene content (6.25 μg/g), followed by P_6-178 (6.24μg/g) and P_6-23 (6.23μg/g); whereas BC_2F_3-CB7-derived seeds of P_7-29 were found with higher β-carotene content (6.80 μg/g) followed by P_7-158 (6.79 μg/g).

Seeds with higher content of tryptophan and β-carotene (CB6: P_6-3 and P_6-69; CB7: P_7-29) were named as PVCBML6 and PVCBML7 and sown during *rabi* 2015–16 to generate BC_2F_3 population. A total of 60 plants for each BC_2F_3 population (PVCBML6 and PVCBML7) were tagged and FG selection was performed for *o2* and *crtRB1-3'TE* loci (Table 1). All plants were found to possess recurrent-type allele at *o2* loci. At *crtRB1-3'TE* loci, one plant was found with RP-type allele, the possible reason for this could be a contamination by outcrossing with pollens of RP. Plants possessing recurrent-type allele at *o2* and donor-type allele *crtRB1-3'TE* loci were subjected for plant phenotyping. The DUS test guidelines for maize suggested by protection of plant varieties and farmer's right authority, India (http://www.plantauthority.gov.in), were used as a reference for phenotyping. In PVCBML6, stem anthocyanin pigmentation was found to be reduced than the original QPM line. Also, the silk color was dark pink in original QPM line which was changed to light pink in PVCBML6 (Fig. 3, online resource 12). In PVCBML7, tassel density was found to be more sparse and tassel was more erect than original QPM line. Silk color was changed to light pink in PVCBML7, which was pure white in original QPM line (Fig. 3, online resource 13). Plants possessing favorable phenotypic characters were selfed to generate BC_2F_4 seeds.

The phenotypic data was recorded on different phenotypic characters and differences in the phenotypic traits between the BC_2F_3 progenies and their RP lines were analyzed using Windostat 8.0 (Table 2). There were non-significant differences in the phenotypic traits between the BC_2F_3 progenies. The tryptophan and lysine content in converted inbreds (PVCBML6 and

Table 2 Statistical analysis

Source	Days to 50% anthesis	Days to 50% silking	Days to maturity	Plant height (cm)	Ear height (cm)	Ear girth (cm)	Ear length (cm)	No. of kernel rows/ear	Number of kernels/row	100 seed weight (g)	Grain yield/plant (g)	Tryptophan content (%)	β-carotene content (μg/g)
PVCBML6	66.47	65.42	112.24	144.17	60.00	13.62	11.48	12.40	21.84	14.99	63.64	0.84	6.20
CBML6	69.59	68.81	112.33	144.13	59.89	13.29	11.58	12.73	21.83	14.68	63.11	0.84	0.71
PVCBML7	71.30	74.55	112.40	159.33	73.92	12.54	13.52	13.88	25.21	20.71	81.79	0.83	6.70
CBML7	71.47	74.41	112.52	159.46	73.67	12.47	13.37	13.75	25.32	20.40	81.59	0.80	1.21
MGU2337	63.85	72.41	111.26	124.27	54.75	12.81	11.83	13.20	22.38	19.84	66.24	0.49	6.14
SEM±	0.44	0.02	0.14	0.34	0.25	0.02	0.22	0.19	0.13	0.23	0.28	2.58	0.02
CD ($p = 0.05$)	1.02	0.05	0.33	0.80	0.58	0.06	0.52	0.45	0.31	0.53	0.65	5.95	0.04
Mean	58.53	71.78	112.15	146.27	64.45	12.95	12.35	13.19	23.31	18.12	71.27	0.76	4.20
CV (%)	0.79	0.04	0.16	0.27	0.48	0.26	2.25	1.83	0.71	1.57	0.48	0.41	0.61

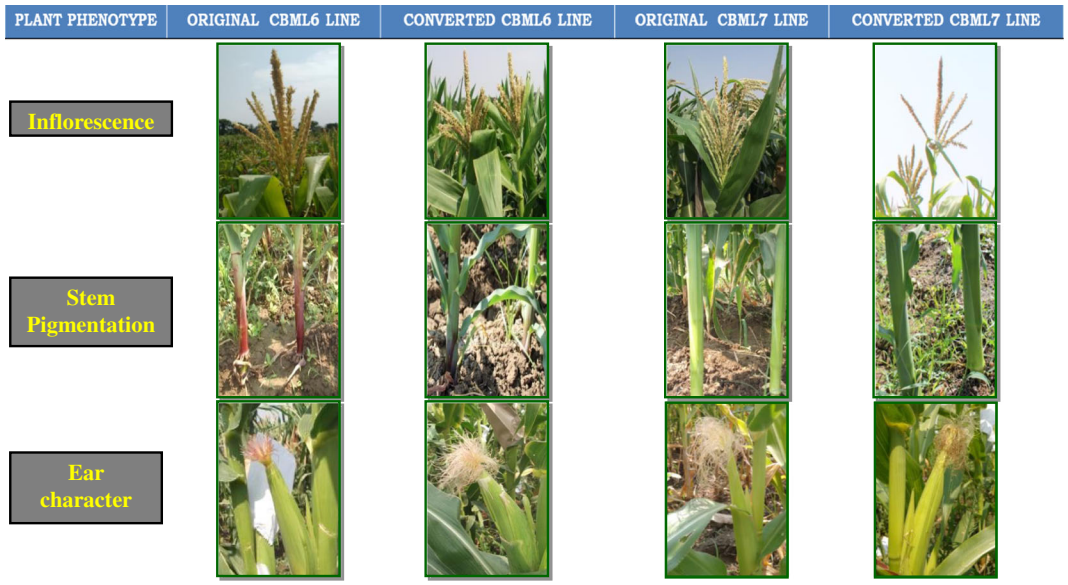

Fig. 3 Plant characters of original and converted version of CBML6 and CBML7

PVCBML7) remained on par with respective RPs. The β-carotene content in converted lines was significantly higher than that of RPs (CB6 and CB7) but not as high as donor (MGU23379).

Discussion

QPM possesses almost the double amount of tryptophan and lysine in kernels than the normal maize (Vasal 2000); however, QPM genotypes also possess very low proA carotenoids (Gupta et al. 2015). Mutant allele of *crtRB1* and *lcyE* significantly enhances proA carotenoids in maize kernels (Harjes et al. 2008; Yan et al. 2010; Babu et al. 2013). The present study used MABB for combining the favorable allele of *crtRB1*-3′TE into QPM inbreds (CBML6 and CBML7—parental lines of popular maize hybrid DHM117). The functional marker for *crtRB1-3′TE* (Yan et al. 2010; Vignesh 2012; Babu et al. 2013; Selvi et al. 2014; Liu et al. 2015; Muthusamy et al. 2015; Zunjare et al. 2017) and *o2*-based *umc1066* marker (Gupta et al. 2013; Hossain et al. 2018; Surender et al. 2017) showed codominance in an earlier investigations. In this study, the functional marker for *crtRB1-3′TE* and *o2*-based *umc1066* marker showed co-dominant polymorphisms between the DP and the RP lines, allowing us to trace the favorable *crtRB1-3′TE* and *umc1066* alleles in each segregating backcross generation and validate homozygous and heterozygous genotypes.

Previous studies reported severe segregation distortion (SD) for *crtRB1-3′TE* across the generations and crosses (Babu et al. 2013; Muthusamy et al. 2014; Liu et al. 2015; Zunjare et al. 2017). In current study, *crtRB1-3′TE* segregated as per Mendelian inheritance because large enough population was screened for achieving sufficient foreground positive genotypes. In majority of populations, *o2* also segregated as per Mendelian inheritance (Gupta et al. 2013; Hossain et al. 2018) with an exception in one population (Jompuk et al. 2011).

Realizing the importance of the FMs for RC selection to minimize linkage drag, FM *umc1506* and *bnlg1028* were used. With these FMs, linkage drag around *crtRB1* locus was minimized, and *crtRB1* transfer was precisely monitored and it made possible to recover DRCs with *crtRB1-3′TE* allele1. FMs for RC selection to minimize linkage drag in MABB program of *o2* introgression have been reported (Babu et al. 2005; Gupta et al. 2009; Gupta et al. 2013; Singh and Ram 2014; Surender et al. 2017), but none of the earlier investigations for enhancing β-carotene in QPM background have used FMs. For the first time, we have reported the use of FMs for *crtRB1* locus to select recombinants.

In present study, SSR markers used in MABB program resulted in high recovery of RPG in two backcross generations and a continuous increase in genomic

recovery ratio in contrast to the theoretical ratio was observed in each backcross generation (Gupta et al. 2013; Muthusamy et al. 2014; Feng et al. 2015; Liu et al. 2015; Hossain et al. 2018; Yang et al. 2018). The introgressed progenies with high RPG attributed to a high degree of resemblance with their corresponding RPs (Muthusamy et al. 2014; Hossain et al. 2018; Yang et al. 2018).

The variation for lysine and tryptophan among *o2*-introgressed progenies was observed in various studies (Gupta et al. 2013; Hossain et al. 2018; Zunjare et al. 2018) in their MABB programs, due to various modifier loci that affect regulation of amino acid biosynthesis. But in current study, tryptophan/lysine content in introgressed inbreds was on par with respective RPs. And the nutritional benefit of QPM with enhanced lysine and tryptophan was conserved in the MABB-derived lines. The reason for this is selection for recurrent-type alleles at modifier loci in BC_1 and BC_2 progenies along with *o2* allele.

Introgressed inbreds possessed significantly higher β-carotene than their respective RPs, but was not as high as DP. This may be due to the contribution of other genetic loci or minor QTLs apart from allele1 of the *crtRB1* gene to the accumulation of β-carotene (Wong et al. 2004; Chander et al. 2008; Zhou et al. 2012; Kandianis et al. 2013). The *crtRB1* and *lcyE* genes show endosperm-specific expression (Babu et al. 2013); and reduced transcript of mutant *crtRB1* leads to lesser amount of β-hydroxylase, and thus lesser conversion of β-carotene to further pathway components (Vallabhaneni et al. 2009; Yan et al. 2010). The advantage of *crtRB1* and *lcyE* together for proA enhancement over individual effects has been reported (Azmach et al. 2013; Gebremeskel et al. 2018; Zunjare et al. 2018). But the favorable alleles of the both genes (*crtRB1* and *lcyE*) occur in low frequency in the maize germplasm (Azmach et al. 2013; Babu et al. 2013; Muthusamy et al. 2015; Gebremeske et al. 2017). Babu et al. (2013) reported a very strong association between allele 1 of *crtRB1* and the endosperm content of β-carotene and proA concentrations irrespective of the genotypic constitution for *lcyE*. The *crtRB1* has much larger effect than *lcyE* on β-carotene and proA concentrations; thus, MAS for the *crtRB1* locus alone is a reliable strategy for rapidly achieving genetic gains for β-carotene and proA carotenoids in tropical maize breeding programs (Babu et al. 2013). The results of current study showed that the favorable *crtRB1-3′TE* allele significantly improved β-carotene. These findings are in accordance with Liu et al. (2015), Muthusamy et al. (2015), and Zunjare et al. (2018) who attempted introgression of *crtRB1* gene into quality protein maize inbred lines using molecular markers and observed increase in β-carotene content of converted lines than the original QPM lines.

Although a significant increase in the β-carotene content was observed in these converted lines, the β-carotene content is less than the target level 15 μg/g proA carotenoids, which emphasize the need of additional efforts to increase these levels. Therefore, further introgression of genetic loci like *crtRB3*, *CCD1*, and *ZEP1* (Zhou et al. 2012; Suwarno et al. 2015) can be attempted to achieve the target levels of proA carotenoids in maize kernel.

The normal and converted lines of CB6 and CB7 were found to be significant for all the phenotypic characters. Agronomic traits showed the stable expression in BC_2F_3 families, and thus represented the final converted versions of the original recipient lines (Babu et al. 2005). The grain yield of converted inbreds was also on par with the original inbreds. The similarity was due to indirect selection of loci for yield potential and various agronomic traits through BG selection (Gupta et al. 2013; Muthusamy et al. 2014; Hossain et al. 2018; Zunjare et al. 2018).

Conclusion

The study implemented a successful demonstration of MABB coupled with rigorous phenotypic selection for agro-morphological characters. These converted inbreds can be used for reconstitution of DHM117 maize hybrid rich in tryptophan, lysine, and β-carotene, which will alleviate VAD and PEM. Converted lines also provides an ideal platform for stacking number of nutritionally important traits such as enhanced Fe, Zn, vitamin E, folate, ascorbic acids, and low phytate. The QPM lines with improved β-carotene concentration serve as an important breeding material for developing QPM maize hybrids with high β-carotene for people dependent on maize as a major component of their diet. Therefore, the development and production of QPM hybrids employing MABB, with high β-carotene concentrations, is an efficient way to deliver biofortified maize for improved health and development benefits to the people of developing countries.

Acknowledgements First author is thankful to Department of Science and Technology (DST), Govt. of India, for providing INSPIRE fellowship to pursue doctoral studies [IF120799]. All authors are thankful to Institute of Biotechnology and Maize Research Centre, PJTSAU, Hyderabad, for providing lab and field facilities to conduct the research.

Authors' contributions Conception and design of study: SSR and DBS, Development of segregating progenies and molecular analysis: DBS. Morphological characterization: DBS and PS. Phenotyping for kernel quality, biochemical analyses: DBS, MS, and PS. Statistical analyses: PS. Drafting of the manuscript: DBS and PS.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

Azmach G, Gedil M, Menkir A, Spillane C (2013) Marker-trait association analysis of functional gene markers for provitamin A levels across diverse tropical yellow maize inbred lines. BMC Plant Biol 13(1):227. https://doi.org/10.1186/1471-2229-13-227

Babu R, Prasanna BM (2014) Molecular breeding for quality protein maize (QPM). In: Tuberosa R, Frison E (eds) Genomics of plant genetic resources. Springer, Dordrecht, pp 489–505. https://doi.org/10.1007/978-94-007-7575-6_21

Babu R, Nair SK, Kumar A, Venkatesh S, Sekhar JC, Singh NN, Srinivasan G, Gupta HS (2005) Two-generation marker-aided backcrossing for rapid conversion of normal maize lines to quality protein maize (QPM). Theor Appl Genet 111(5):888–897. https://doi.org/10.1007/s00122-005-0011-6

Babu R, Rojas NP, Gao S, Yan J, Pixley K (2013) Validation of the effects of molecular marker polymorphisms in LcyE and CrtRB1 on provitamin A concentrations for 26 tropical maize populations. Theor Appl Genet 126(2):389–399. https://doi.org/10.1007/s00122-012-1987-3

Bain LE, Awah PK, Geraldine N, Kindong NP, Sigal Y, Bernard N, Tanjeko AT (2013) Malnutrition in Sub-Saharan Africa: burden, causes and prospects. Pan Afr Med J 15(1). https://doi.org/10.11604/pamj.2013.15.120.2535

Chander S, Guo YQ, Yang XH, Zhang J, Lu XQ, Yan JB, Song TM, Rocheford TR, Li JS (2008) Using molecular markers to identify two major loci controlling carotenoid contents in maize grain. Theor Appl Genet 116(2):223–233. https://doi.org/10.1007/s00122-007-0661-7

Danson J, Mbogori M, Kimani M, Lagat M, Kuria A, Diallo A (2006) Marker-assisted introgression of opaque2 gene into herbicide tolerant elite maize inbred lines. Afr J Biotechnol 5: 2417–2422 Available at: http://hdl.handle.net/10883/3032

Feng F, Wang Q, Liang C, Yang R, Li X (2015) Enhancement of tocopherols in sweet corn by marker-assisted backcrossing of ZmVTE4. Euphytica 206(2):513–521. https://doi.org/10.1007/s10681-015-1519-8

Gebremeskel S, Garcia-oliveira AL, Menkir A, Adetimirin V, Gedil M (2018) Effectiveness of predictive markers for marker assisted selection of pro-vitamin A carotenoids in medium-late maturing maize (Zea mays L.) inbred lines. J Cereal Sci 79:27–34. https://doi.org/10.1016/j.jcs.2017.09.001

Gupta HS, Hossain F, Muthusamy V (2015) Biofortification of maize: an Indian perspective. Indian J Genet Plant Breed 75(1):1–22. https://doi.org/10.5958/0975-6906.2015.00001.2

Gupta HS, Raman B, Agrawal PK, Mahajan V, Hossain F, Thirunavukkarasu N (2013) Accelerated development of quality protein maize hybrid through marker-assisted introgression of opaque-2 allele. Plant Breed 132(1):77–82. https://doi.org/10.1111/pbr.12009

Gupta HS, Agrawal PK, Mahajan V, Bisht GS, Kumar A, Verma P et al (2009) Quality protein maize for nutritional security: rapid development of short duration hybrids through molecular marker-assisted breeding. Curr Sci 96:230–237 http://www.jstor.org/stable/24105185

Harjes CE, Rocheford TR, Bai L, Brutnell TP, Kandianis CB, Sowinski SG, Stapleton AE, Vallabhaneni R, Williams M, Wurtzel ET, Yan J, Buckler ES (2008) Natural genetic variation in lycopene epsilon cyclase tapped for maize biofortification. Science 319(5861):330–333. https://doi.org/10.1126/science.1150255

Hernandez HH, Bates LS (1969) A modified method for a rapid tryptophan analysis in maize. CIMMYT, Mexico City, Mexico, Res Bull 13:7 Available at: https://hdl.handle.net/10883/19403

Hossain F, Muthusamy V, Pandey N, Vishwakarma AK, Baveja A, Zunjare RU, Thirunavukkarasu N, Saha S, Manjaiah KM, Prasanna BM, Gupta HS (2018) Marker-assisted introgression of opaque2 allele for rapid conversion of elite hybrids into quality protein maize. J Genet 97(1):287–298. https://doi.org/10.1007/s12041-018-0914-z

Jompuk C, Cheuchart P, Jompuk P, Apisitwanich S (2011) Improved tryptophan content in maize with opaque-2 gene using marker assisted selection (MAS) in backcross and selfing generations. Kasetsart J Nat Sci 45:666–674 Available at: www.researchgate.net

Kandianis CB, Stevens R, Liu W, Palacios N, Montgomery K, Pixley K, White WS, Rocheford T (2013) Genetic architecture controlling variation in grain carotenoid composition and concentrations in two maize populations. Theor Appl Genet 126(11):2879–2895. https://doi.org/10.1007/s00122-013-2179-5

Kennedy G, Nantel G, Shetty P (2003) The scourge of hidden hunger: global dimensions of micronutrient deficiencies. Food Nutr Agric 32:8–16 Available at: http://www.fao.org/docrep/005/Y8346M/y8346m02.htm

Kumar RS, Kumar B, Kaul J, Karjagi CG, Jat SL, Parihar CM et al (2012) Maize research in India-historical prospective and future challenges. Maize Journal 1:1–6 Available at: www.researchgate.net

Liu L, Jeffers D, Zhang Y, Ding M, Chen W, Kang MS, Fan X (2015) Introgression of the *crtRB1* gene into quality protein maize inbred lines using molecular markers. Mol Breed 35(8):154. https://doi.org/10.1007/s11032-015-0349-7

Mertz ET, Bates LS, Nelson OE (1964) Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science 145(3629):279–280. https://doi.org/10.1126/science.145.3629.279

Muthusamy V, Hossain F, Thirunavukkarasu N, Choudhary M, Saha S, Bhat JS, Prasanna BM, Gupta HS (2014) Development of β-carotene rich maize hybrids through marker-assisted introgression of β-carotene hydroxylase allele. PLoS One 9(12):e113583. https://doi.org/10.1371/journal.pone.0113583

Muthusamy V, Hossain F, Thirunavukkarasu N, Saha S, Gupta HS (2015) Allelic variations for lycopene-ε-cyclase and β-carotene hydroxylase genes in maize inbreds and their utilization in β-caroteneenrichment programme. Cogent Food Agric 1(1033141). https://doi.org/10.1080/23311932.2015.1033141

Neeraja CN, Ravindra Babu V, Ram S, Hossain F, Hariprasanna K, Rajpurohit BS, Prabhakar, Longvah T, Prasad KS, Sandhu JS, Datta SK (2017) Biofortification in cereals : progress and prospects. Curr Sci 113(6):1050–1057. https://doi.org/10.18520/cs/v113/i06/1050-1057

Nuss ET, Tanumihardjo SA (2010) Maize: a paramount staple crop in the context of global nutrition. Compr Rev Food Sci Food Saf 9(4):417–436. https://doi.org/10.1111/j.1541-4337.2010.00117.x

Nyakurwa CS, Gasura E, Mabasa S (2017) Potential for quality protein maize for reducing protein energy undernutrition in maize dependent Sub-Saharan African countries: a review. Afr Crop Sci J 25(4):521–537. https://doi.org/10.4314/acsj.v25i4.9

Rodriguez-Amaya DB (2001) A guide to carotenoid analysis in foods. Washington, ILSI press

Sagare DB, Shetti P, Surender M, Reddy SS, Pradeep T, Anuradha G (2018) Maize: potential crop for provitamin A biofortification. Maydica 63(2):11 Available at: https://journals-crea.4science.it/index.php/maydica/article/view/1645

Selvi DT, Senthil N, Yuvaraj A, Joel AJ, Mahalingam A, Nagarajan P, Vellaikumar S, Srimathi P, Raveendran M, Nepolean T (2014) Assessment of *crtRB1* polymorphism associated with increased β-carotene content in maize (*Zea mays* L.) seeds. Food Biotechnol 28(1):41–49. https://doi.org/10.1080/08905436.2013.870077

Singh R, Ram L (2014) DNA Aided Introgression of *opaque2* allele for development of quality protein maize. Int J Sci Res 3(11): 3006–3011 Available at: googlescholar

Surender M (2015) Conversion of elite non QPM inbreds of maize to QPM using marker assisted selection. Ph.D. In: Thesis. Professor Jayashankar Telangana State Agricultural University, Hyderabad Available at: http://krishikosh.egranth.ac.in/handle/1/92608

Surender M, Shetti P, Sagare DB, Rani CV, Jabeen F, Sudarshan MR et al (2017) Development of QPM version of DHM117 maize hybrid using marker assisted selection. Int J Curr Microbiol Appl Sci 6(10):3275–3289. https://doi.org/10.20546/ijcmas.2017.610.384

Suwarno WB, Pixley KV, Palacios-Rojas N, Kaeppler SM, Babu R (2015) Genome-wide association analysis reveals new targets for carotenoid biofortification in maize. Theor Appl Genet 128(5):851–864. https://doi.org/10.1007/s00122-015-2475-3

Tanumihardjo SA, Anderson C, Kaufer-Horwitz M, Bode L, Emenaker NJ, Haqq AM, Satia JA, Silver HJ, Stadler DD (2007) Poverty, obesity, and malnutrition: an international perspective recognizing the paradox. J Am Diet Assoc 107(11):1966–1972. https://doi.org/10.1016/j.jada.2007.08.007

Vallabhaneni R, Gallagher CE, Licciardello N, Cuttriss AJ, Quinlan RF, Wurtzel ET (2009) Metabolite sorting of a germplasm collection reveals the hydroxylase3 locus as a new target for maize provitamin A biofortification. Plant Physiol 151(3):1635–1645. https://doi.org/10.1104/pp.109.145177

Vasal SK (2000) The quality protein maize story. Food Nutr Bull 21(4):445–450. https://doi.org/10.1177/156482650002100420

Vignesh M, Hossain F, Nepolean T, Supradip S, Agrawal PK, Guleria SK, et al (2012) Genetic variability for kernel beta-carotene and utilization of crtRB1 3'TE gene for biofortification in maize (*Zea mays* L.). Indian J Genet Plant Breed 72: 189–194 Available at: http://hdl.handle.net/10883/3175

Vivek BS, Krivanek AF, Palacios-Rojas N, Twumasi-Afriyie S, Diallo AO (2008) Breeding quality protein maize (QPM): protocols for developing QPM cultivars. Mexico: CIMMYT Available at: http://hdl.handle.net/10883/801

Wong JC, Lambert RJ, Wurtzel ET, Rocheford TR (2004) QTL and candidate genes phytoene synthase and zeta-carotene desaturase associated with the accumulation of carotenoids in maize. Theor Appl Genet 108(2):349–359. https://doi.org/10.1007/s00122-003-1436-4

Yan J, Kandianis CB, Harjes CE, Bai L, Kim EH, Yang X, Skinner DJ, Fu Z, Mitchell S, Li Q, Fernandez MGS, Zaharieva M, Babu R, Fu Y, Palacios N, Li J, DellaPenna D, Brutnell T, Buckler ES, Warburton ML, Rocheford T (2010) Rare genetic variation at Zea mays crtRB1 increases beta-carotene in maize grain. Nat Genet 42(4):322–327. https://doi.org/10.1038/ng.551

Yang R, Yan Z, Wang Q, Li X, Feng F (2018) Marker-assisted backcrossing of lcyE for enhancement of proA in sweet corn. Euphytica 214(8):130. https://doi.org/10.1007/s10681-018-2212-5

Zhang X, Pfeiffer WH, Palacios-Rojas N, Babu R, Bouis H, Wang J (2012) Probability of success of breeding strategies for improving pro-vitamin A content in maize. Theor Appl Genet 125(2):235–246. https://doi.org/10.1007/s00122-012-1828-4

Zhou Y, Han Y, Li Z, Fu Y, Fu Z, Xu S, Li J, Yan J, Yang X (2012) ZmcrtRB3 encodes a carotenoid hydroxylase that affects the accumulation of α-carotene in maize kernel. J Integr Plant Biol 54(4):260–269. https://doi.org/10.1111/j.1744-7909.2012.01106.x

Zunjare RU, Hossain F, Muthusamy V, Baveja A, Chauhan HS, Thirunavukkarasu N, Saha S, Gupta HS (2017) Influence of rare alleles of β-carotene hydroxylase and lycopene epsilon cyclase genes on accumulation of provitamin A carotenoids in maize kernels. Plant Breed 136(6):872–880. https://doi.org/10.1111/pbr.12548

Zunjare RU, Hossain F, Muthusamy V, Baveja A, Chauhan HS, Bhat JS, Thirunavukkarasu N, Saha S, Gupta HS (2018) Development of biofortified maize hybrids through marker-assisted stacking of β-*carotene hydroxylase, lycopene-ε-cyclase* and *opaque2* genes. Front Plant Sci 9(178). https://doi.org/10.3389/fpls.2018.00178