



Enhancing β -Carotene Concentration in Parental Lines of CO6 Maize Hybrid Through Marker-Assisted Backcross Breeding (MABB)

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Vitamin A deficiency (VAD) is a global health problem; many people around the world, especially children and pregnant women, are VAD deficient or insufficient. Maize is known as an important source of provitamin A for humans. Hence, enhancement of provitamin A carotenoids (pVAC) in maize varieties through breeding or biofortification is a good option for alleviating VAD in developing countries, especially India. So far, numerous maize hybrids have been developed in India. Among them, CO6, derived from UMI1200 \times UMI1230, is a popular maize hybrid and adapted to different agro-climatic zones of India, especially Tamil Nadu, a southern state of India. However, CO6 is deficient for pVAC carotenoid β -carotene. Thus, the objectives of this study were to increase the β -carotene concentration in UMI1200 and UMI1230 and generate the β -carotene enriched hybrids through marker-assisted backcross breeding (MABB). For this purpose, the maize genotype HP467-15 was used as the donor for transferring the β -carotene gene, *crtRB1*, into UMI1200 and UMI1230. In the MABB scheme, we used one gene-specific marker (*crtRB1* 3'TE) and 214 simple sequence repeat (SSR) markers for foreground and background selection, respectively. As a result, six improved lines with recurrent parent genome recovery (RPGR) ranging from 90.24 to 92.42%, along with good agronomic performance, were generated. The β -carotene concentration of the improved lines ranged from 7.056 to 9.232 $\mu\text{g/g}$. Furthermore, five hybrid combinations were generated using improved lines and evaluated in a comparative yield trial (CYT) and multi-location trials (MLT) along with the original hybrid CO6 and commercial hybrids. It was revealed that ACM-M13-002 was a superior hybrid with a 7.3-fold increase in

β -carotene concentration and with a comparable yield to CO6. In summary, the improved maize inbreds can be used as possible donors for the development of β -carotene-rich cultivars in maize breeding programs and the β -carotene enriched hybrid developed in this study will hold great promise for food and nutritional security.

Keywords: β -Carotene, Co6 maize hybrid, *crtRB1*, vitamin A deficiency, MABB

INTRODUCTION

Vitamin A deficiency (VAD) is one of the most prevalent micronutrient deficiencies and poses a serious health problem to children and pregnant women around the world, especially in developing countries. The consequences of VAD include night blindness, reduced growth in children, and increased morbidity and mortality (1). Maize (*Zea mays* L.) is one of the major sources of provitamin A for humans. Thus, the improvement of provitamin A carotenoids (pVAC) in maize varieties through breeding or biofortification is a successful and cost-effective approach for alleviating VAD in developing countries, especially India (2). The preference of maize for biofortification also relies on higher bioavailability and bioconversion of pVAC into vitamin A. α -carotene, β -carotene, β -cryptoxanthin, zeaxanthin, and lutein are important carotenoid compounds that exist in maize grains (3). Among them, β -carotene has the highest provitamin A activity and can be converted easily to vitamin A by the human metabolism (4, 5). However, the success of this approach depends on the identification of superior maize genotypes with a high β -carotene concentration by characterizing the maize genotypes and subsequently transferring the high β -carotene from the superior genotypes to the popular maize cultivars through introgression breeding. However, β -carotene is present at a comparatively low level in maize kernels of popular cultivated lines, and only a very small percentage of maize varieties have naturally high β -carotene levels. Muthusamy et al. screened 105 maize inbreds of both Indian and CIMMYT origins for kernel β -carotene content. The results showed that kernel β -carotene concentration varied from 0.02 to 16.50 $\mu\text{g/g}$ in maize inbreds (6). Hence, there is a need to understand the major genes involved in the carotenoid biosynthesis pathway that could be modulated for enhancing β -carotene concentration.

Recent advancements in maize genomics have reduced the breeding cycles and significantly increased the precision and effectiveness of breeding methods, and are also helping to identify the genes and biosynthesis pathways involved in β -carotene accumulation. Maize breeding programs through marker-assisted selection (MAS) have become more standard with the availability of reasonably dense molecular marker linkage maps, and many researchers have been pursuing the association of markers with known genes. Lycopene epsilon cyclase (*lcyE*) located at chromosome 8 and β -carotene hydroxylase enzyme (*crtRB1*) located at chromosome 10 are the two major genes associated with β -carotene accumulation in maize (3, 7). Among them, the gene *crtRB1* was stated to have a much greater effect on the concentrations of β -carotene than that of *lcyE* (8). The favorable allele, namely *crtRB1* 3'TE (allele 1, 543 bp), is

accountable for causing a 2- to 10-fold upsurge in β -carotene concentration (8). Polymerase chain reaction (PCR) based co-dominant markers from the 3'TE region of the *crtRB1* gene provided opportunities to identify and develop the higher β -carotene concentration genotypes through MAS. Several studies showed the feasibility of this allele-based marker to detect the *crtRB1* allele in diverse maize genotypes (9–11).

In India, maize is one of the major cereal crops after rice and wheat, and is utilized as a major source of both food and feed. To date, several maize inbreds and hybrids have been developed for their economically important traits and are commercially available in India. Among them, CO6, derived from UMI1200 \times UMI1230, is a popular single-cross hybrid and adapted to different agro-climatic zones of India, especially Tamil Nadu, a southern state of India. CO6 is well-suited for cultivation in both under irrigate and rainfed conditions and also exhibits multiple disease resistances. It shows superior performance in grain yield and quality, and ease in hybrid seed production (12). These salient features make CO6 a popular maize hybrid among farmers. However, CO6 is deficient in nutritional traits, especially β -carotene. Thus, it is necessary to improve the CO6 hybrid with a higher β -carotene concentration. Marker-assisted backcross breeding (MABB) is an effective approach to improve the nutritional traits in maize. Recently, several studies showed success with improving the nutritional traits in maize hybrids (Pusa Vivek QPM9 Improved, Pusa HM4 Improved, Pusa HM8 Improved, and Pusa HM9 Improved) using MABB (9, 13). With this background information, the present study aimed to: (i) introgress the *crtRB1* allele into the parental lines (UMI1200 and UMI1230) of CO6 hybrid using MABB; (ii) evaluate the improved lines agronomic performance and β -carotene concentration; and (iii) generate the hybrids using the improved lines and evaluate the hybrids in diverse maize growing regions of Tamil Nadu, a southern state of India.

MATERIALS AND METHODS

Plant Genetic Materials

CO6 is a popular maize hybrid well-adapted to different agro-climatic zones of India, especially Tamil Nadu, a southern state of India. Maize inbreds viz., UMI1200 and UMI1230 are the parental lines of the CO6 hybrid. UMI1200 is a female parent for the CO6 hybrid and is suitable for tropical regions; it is characterized as a dent grain type. UMI1230 is a pollen parent for the CO6 hybrid and is suitable for both tropical and semi-arid regions. It is characterized as a flint type. Both have diverse genetic backgrounds and are placed in different subspecies in

accordance with the starch type in the endosperm. Due to their excellent combining ability, these two inbreds were used to develop the CO6 hybrid. The donor parent was HP467-15 from CIMMYT (International Maize and Wheat Improvement Center, Mexico), which carries the *crtRB1* allele responsible for the high β -carotene concentration.

Outline of the Conversion of Maize Inbreds to Its High β -Carotene Version

The conversion process included crossing, two generations of backcrossing, and two generations of selfing. All the field experiments were carried out on the Central farm, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India from 2011 to 2015. Foreground selection was done using a *crtRB1* gene-specific marker (*crtRB1* 3'TE) (7) to select the plants at F₁, backcross, and selfed generations. Foreground selection targeted heterozygous plants with allele 1, 543 bp (Favorable allele) and allele 3, 296 bp (Unfavorable allele), or homozygous plants with allele 1, 543 bp (Favorable allele). Background selection was done to select the foreground positive plants with maximum recurrent parent genome recovery (RPGR) using polymorphic simple sequence repeat (SSR) markers in backcross and selfed generations. Two independent crosses, namely UMI1200 \times HP467-15 and UMI1230 \times HP467-15, were made during Rabi 2011-12 to produce F₁ seeds. In Kharif 2012, the F₁ plants were confirmed for their heterozygosity and backcrossed with the recurrent parent to produce BC₁F₁ progenies. In Rabi, 2012-13, the BC₁F₁ plants that were found to be heterozygous were backcrossed with the recurrent parent to produce BC₂F₁ progenies. Further, BC₂F₁ plants were screened to identify heterozygous and the selected plants were selfed to produce BC₂F₂ progenies during Kharif 2013. In Rabi 2013-14, BC₂F₂ progenies were screened before flowering to identify plants with allele 1, 543 bp (Favorable allele) and the selected plants were selfed to produce BC₂F₃ lines, whereas the homozygous plants with allele 3, 296 bp (Unfavorable allele) and heterozygous plants with allele 1, 543 bp (Favorable allele) and allele 3, 296 bp (Unfavorable allele) were excluded. Agro-morphological character evaluation and β -carotene estimation were carried out in BC₂F₃ improved lines.

Genomic DNA Isolation and Marker Genotyping

The genomic DNA was isolated from the young leaves using the cetyl trimethyl ammonium bromide (CTAB) method (14). The DNA was checked for its quantity and quality on 0.8% agarose gel. The *crtRB1* gene-specific marker (*crtRB1* 3'TE) (65F: ACACCACATGGACAAGTTCG, 62R: AACTCTGGCCCATG AACAC, 66R: ACAGCAATACAGGGACCAG) was used for foreground selection. A total of 214 simple sequence repeat (SSR) markers spanning uniformly across the maize genome were used for background selection. The primer sequences for SSR markers were obtained from the maize genome database (www.maizegdb.org) and were synthesized by Eurofins Ltd, Bangalore, India. The polymerase chain reaction (PCR) for *crtRB1* 3'TE/SSR markers and gel electrophoresis were carried out following the procedure

of Muthusamy et al. and Pukalenty et al. (9, 15). All the obtained genotyping results were tested for goodness of fit using the chi-square analysis. The amplicons of the markers were scored as "A" for the recurrent parent, "B" for the donor parent, and "H" for heterozygotes. Chi-square analysis was done using PROC MIXED of SAS and LSMEANS and ADJUST options were used to find the least square mean values and for the comparison of the *p*-value. The percentage of RPGR was calculated following the procedure of Sundaram et al. (16).

Evaluation of Agro-Morphological Characters in Improved Lines

The recurrent and donor parents and the improved lines were evaluated for eight agro-morphological characters [plant height (cm), days to 50% tasselling (days), days to 50% silking (days), cob length (cm), number of kernel rows/ cob, number of kernels/row, grain yield/ plant (g), and 100-kernel weight (g)] which were recorded according to the plant stage based on the standard maize descriptors formulated by IBPGR (17).

Estimation of β -Carotene Concentration Using HPLC Analysis

The seeds obtained from the recurrent and donor parents and the improved lines were subjected to β -carotene estimation. Since the carotenoid compounds are sensitive to light, the seeds were harvested at 14% moisture content and stored at 22–26°C. Estimation of β -carotene was carried out by adopting the Harvest plus protocol (18). The carotenoid compounds were extracted by grinding the seed samples using ice-cold acetone until a fine powder was obtained. Since the carotenoid compounds undergo photo-oxidation, further processing was done under yellow light (19, 20). The samples extracted were concentrated in a rotary evaporator at 45°C and made up to 2 ml using methanol prior to separation. The β -carotene concentration was quantified by high-performance liquid chromatography (HPLC). Samples were eluted by C18G 120A column (250 \times 4.6 mm) and measured with a photodiode array detector set at 450 nm. The mobile phase comprised of Acetonitrile: Methanol: Ethyl acetate (80:10:10) at high pressure by the column with the flow rate of 1 mL min⁻¹. β -carotene (M/s. Sigma Aldrich, India) reconstituted in methanol to five different concentrations (0.1 μ g/g; 1 μ g/g; 10 μ g/g; 50 μ g/g; 100 μ g/g) was used to make the standard curve. The β -carotene concentration was identified by their characteristic spectra and comparison of their retention times with known standard solutions.

Generation of Hybrids Using Improved Lines and Their Evaluation

Four improved lines from UMI1200 \times HP467-15 and two improved lines from UMI1230 \times HP467-15 were crossed during Kharif 2014 to generate the five hybrids. These hybrids were evaluated along with the commercial hybrid NK6240 and CO6 during the Rabi 2014-15 and Kharif 2015 at the Experimental Farm, Agricultural Research Station, Vaigai dam, Tamil Nadu Agricultural University, India and Maize Research Station, Tamil Nadu Agricultural University, Vagarai, India under comparative

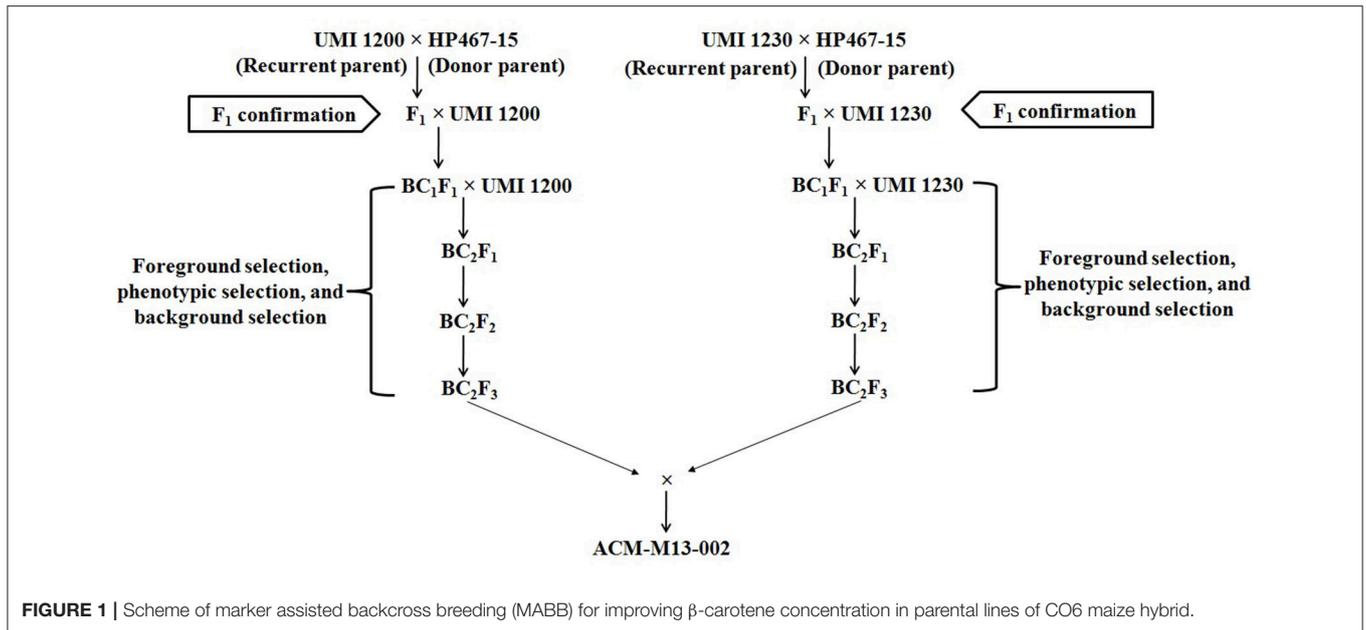


FIGURE 1 | Scheme of marker assisted backcross breeding (MABB) for improving β -carotene concentration in parental lines of CO6 maize hybrid.

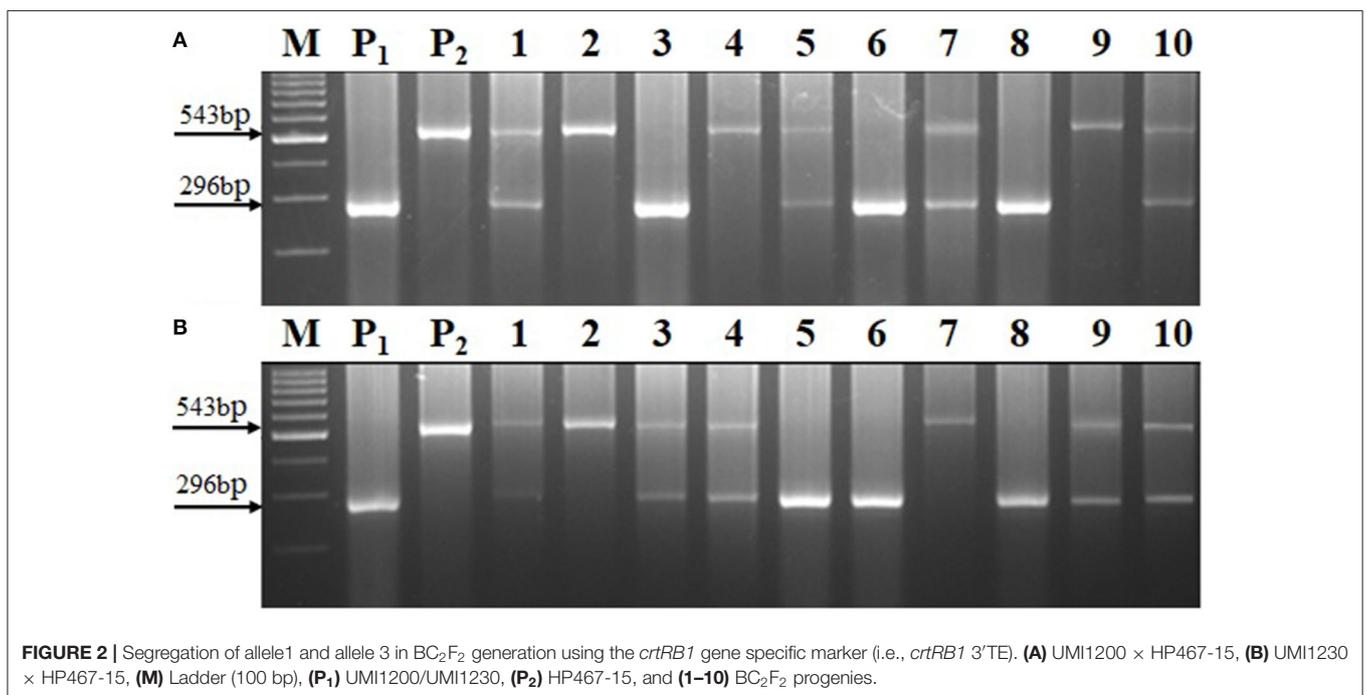


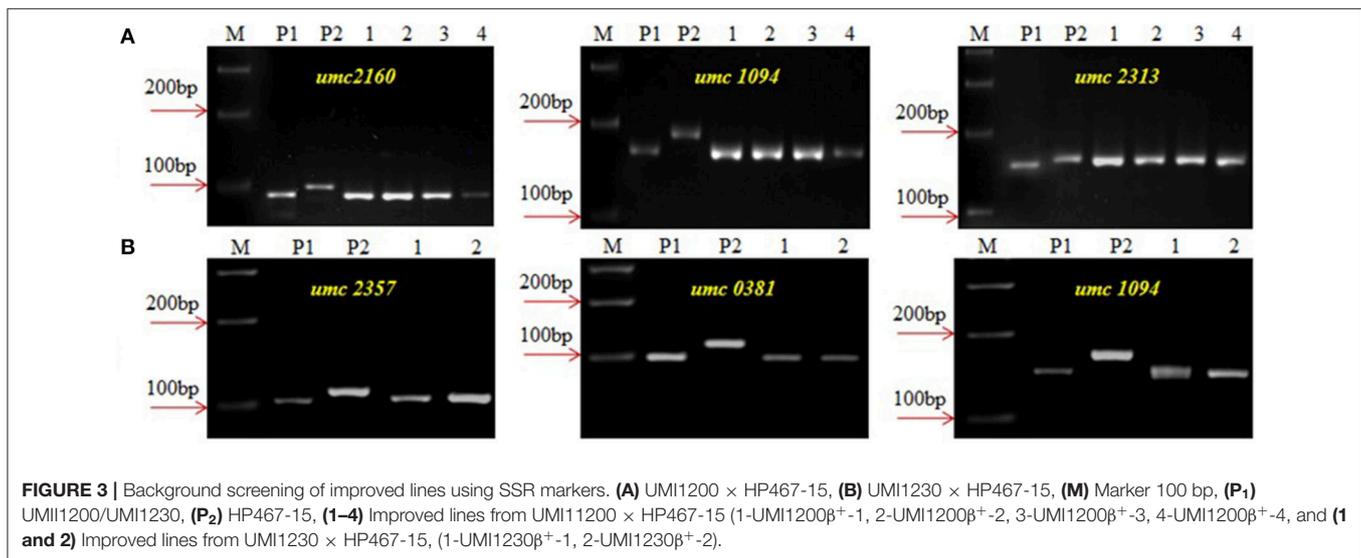
FIGURE 2 | Segregation of allele1 and allele 3 in BC₂F₂ generation using the *crtRB1* gene specific marker (i.e., *crtRB1* 3'TE). **(A)** UMI1200 × HP467-15, **(B)** UMI1230 × HP467-15, **(M)** Ladder (100 bp), **(P₁)** UMI1200/UMI1230, **(P₂)** HP467-15, and **(1–10)** BC₂F₂ progenies.

yield trial (CYT). Further, one superior hybrid identified from CYT was tested in multi-location trials (MLT) during Kharif 2016 and 2017 in diverse maize growing environments of Tamil Nadu along with eight maize hybrids including commercial hybrids. Kharif 2016 included four environments viz., Coimbatore (E1), Vagarai (E2), Vridhachalam (E3), and Bhavanisagar (E4) and Kharif 2017 included six environments viz., Coimbatore (E5), Vagarai (E6), Vridhachalam (E7), Bhavanisagar (E8), Vaigai Dam (E9), and Athiyandal (E10). Hybrids [namely NK6240 (G1), CO 6 (G2), ACM-M13-002 (G3), ACM-M13-007 (G4),

CMH 11-583 (G5), VaMH 14020 (G6), VaMH 12014 (G7), 900 M GOLD (G8), and CMH 11-586 (G9)] were used in MLT. All the field trials were conducted in the irrigated condition. The spacing followed was 60 × 25 cm with a plot size of 5 × 3.6 m (6 rows each) and a fertilizer dosage of 250:75:75 kg/ha. Trials were carried out using a randomized complete block design (RCBD) with two replications. Grain yield data were subjected to a combined analysis of variance (ANOVA) using GEA-R statistical software (21). Additive Main Effect and Multiplicative Interaction (AMMI) model (22) and Genotype

TABLE 1 | Segregation pattern of alleles of the *crtRB1* gene in backcrossed and selfed progenies.

Cross	Generation	Population size	No of homozygotes (allele1/allele1)	No of heterozygotes (allele1/allele3)	No of homozygotes (allele3/allele3)	χ^2	P-value
1 UMI1200 \times HP467-15	BC ₁ F ₁	100	–	24	76	27.04	0.0001*
	BC ₂ F ₁	53	–	11	42	18.13	0.0001*
	BC ₂ F ₂	146	34	46	66	33.99	0.0004*
2 UMI1230 \times HP467-15	BC ₁ F ₁	100	–	37	63	6.76	0.0093*
	BC ₂ F ₁	87	–	18	69	29.89	0.0005*
	BC ₂ F ₂	126	31	67	28	14.25	0.0008*

*Significant at $P < 0.05$.

and Genotype by Environmental Interaction (GGE) effects biplot (23) were employed to analyse the Genotype by Environment (GE) interaction and to assess grain yield stability based on the principal component analysis (PCA).

RESULTS

Marker-Assisted Introgression of the *crtRB1* Gene Into UMI1200 and UMI1230

The *crtRB1* allele was introgressed into UMI1200 and UMI1230 using a recurrent backcrossing procedure, combined with foreground and background selection (Figure 1). Parental polymorphism screening was conducted between the recurrent and donor parents using a set of 214 SSR markers. In the cross combinations UMI1200 \times HP467-15 and UMI1230 \times HP467-15, among the 214 SSR markers, 117 and 109 SSR markers were found to be polymorphic markers. These polymorphic SSR markers were used for background selection and RPGR analysis. The F₁ plants were produced from crosses UMI1200 \times HP467-15 and UMI1230 \times HP467-15. After confirming heterozygosity with a *crtRB1* 3' TE marker, the true F₁ plants were backcrossed to their respective recurrent parent. The resulting BC₁F₁ population were screened with a *crtRB1* 3' TE marker and 24 heterozygous

plants (allele 1/allele 3) in UMI1200 \times HP467-15 and 37 heterozygous plants (allele 1/allele 3) in UMI1230 \times HP467-15 were identified. The percentage of RPGR of positive plants ranged from 66.52–78.75% to 66.41–78.43%. The best BC₁F₁ plant showed a maximum RPGR of 78.75 and 78.43% was further selected to produce the BC₂F₁ population. In the BC₂F₁ population, a total of 53 and 87 plants were screened with a *crtRB1* 3' TE marker. Eleven heterozygous (allele 1/allele 3) plants in UMI1200 \times HP467-15 and 18 heterozygous plants (allele 1/allele 3) in UMI1230 \times HP467-15 were identified in the BC₂F₁ population. The percentage of RPGR of positive plants ranged from 81.39–88.12% to 82.59–89.65%. The best three BC₂F₁ plants from each cross having maximum RPGR were further selected and selfed to produce the BC₂F₂ population. A total of 146 and 126 BC₂F₂ plants were screened with a *crtRB1* 3' TE marker to identify homozygous plants (allele 1) (Figure 2). It revealed 34 homozygous plants in UMI1200 \times HP467-15 and 31 homozygous plants in UMI1230 \times HP467-15. The percentage of RPGR of positive plants ranged from 87.26–92.42% to 88.36–92.21%. The population details and their segregation pattern are presented in Table 1. The numbers of selected plants were further reduced to four and two based on the RPGR and phenotype data, and selfed to produce BC₂F₃ lines. As a result, four BC₂F₃

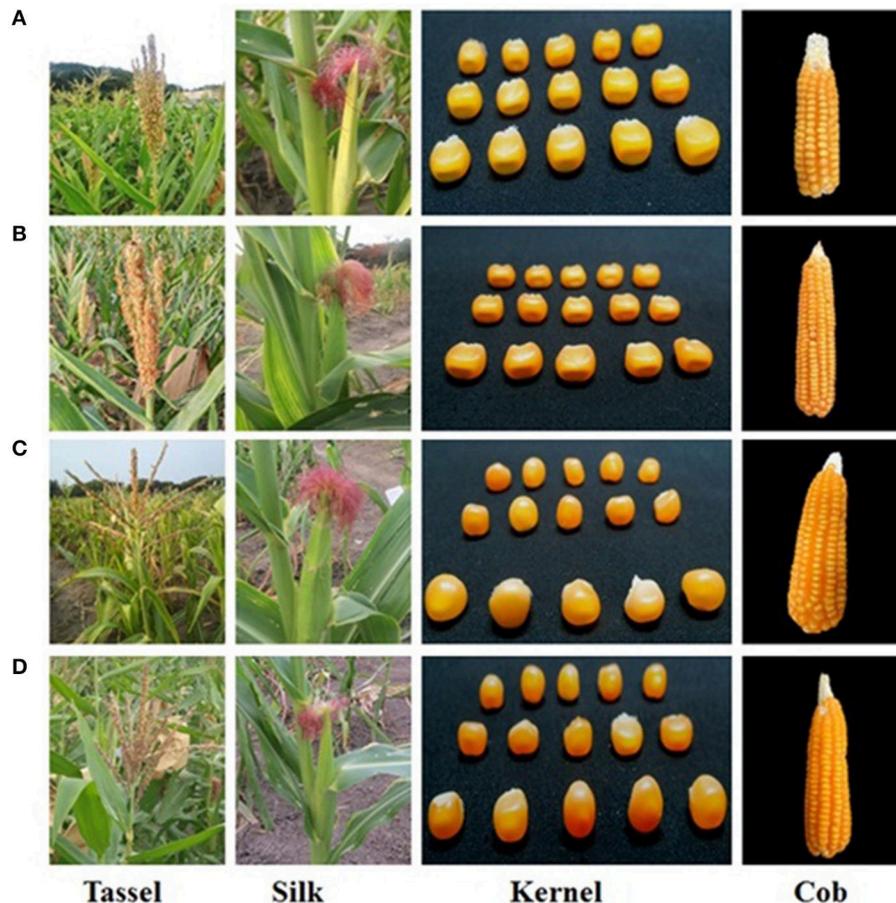


FIGURE 4 | Morphological resemblance of the parents and the improved lines. **(A)** UMI1200, **(B)** UMI1200 β^+ -1, **(C)** UMI1230, **(D)** UMI1230 β^+ -1.

improved lines (UMI1200 β^+ -1, UMI1200 β^+ -2, UMI1200 β^+ -3, and UMI1200 β^+ -4) from the cross UMI1200 \times HP467-15 and two BC₂F₃ improved lines (UMI1230 β^+ -1 and UMI1230 β^+ -2) from the cross UMI1230 \times HP467-15 were developed. UMI1200 \times HP467-15 based four BC₂F₃ lines showed 90.24–92.42% RPGR with an average of 91.33% and UMI1230 \times HP467-15 based two BC₂F₃ lines showed 90.41% to 92.21% RPGR with an average of 91.31%. Among the improved lines, UMI1200 β^+ -1 and UMI1230 β^+ -1 showed the highest RPGR of 92.42% and 92.21% from their respective crosses (Figure 3). The positive plant's RPGR details are presented in Supplementary Tables 1, 2.

Introduction of *crtRB1* Gene Had No Effects on Agro-Morphological Characters

Six improved lines from both crosses were evaluated for the agro-morphological characters (Figure 4 and Table 2). It was revealed that all the improved lines recorded more than 80% of phenotypic resemblance, except for the number of kernel rows per cob (71.43%), to their respective recurrent parent. Among them, UMI1200 β^+ -1, UMI1200 β^+ -2, and UMI1200 β^+ -4 from UMI1200 \times HP467-15 and UMI1230 β^+ -1 from UMI1230 \times HP467-15 recorded high phenotypic resemblance percentage

(>90%) for more than six and five traits *viz.*, days to 50% tasselling, days to 50% silking, plant height, cob length, number of kernels per row, 100 kernel weight, and grain yield per plant. The overall performance showed that the traits recorded among the improved lines were on par with their recurrent parents and its phenotypic resemblance percentage ranging from 71.43% (number of kernel rows per cob) to 99.89% (grain yield per plant).

β -Carotene Concentration in Improved Lines

The β -carotene concentration was estimated in six improved lines; it showed an increased level of β -carotene ranging from 7.056 to 9.232 $\mu\text{g/g}$ with an average of 8.322 $\mu\text{g/g}$. The β -carotene concentration for the recurrent parents was 0.893 $\mu\text{g/g}$ (UMI1200) and 1.610 $\mu\text{g/g}$ (UMI1230). Introgression of allele1 resulted in an increased level of the kernel β -carotene concentration with a maximum of 9.073 $\mu\text{g/g}$ in UMI1200 β^+ -1 from UMI1200 \times HP467-15 and 9.232 $\mu\text{g/g}$ in UMI1230 β^+ -1 from UMI1230 \times HP467-15. We also recorded a minimum level of the kernel β -carotene concentration 7.056 $\mu\text{g/g}$ in UMI1200 β^+ -3 and 9.021 $\mu\text{g/g}$ in UMI1230 β^+ -2. However, it was higher than their respective recurrent parent.

TABLE 2 | β -carotene concentration and agronomic performance of the improved lines developed through MABB.

Genotypes	β -carotene ($\mu\text{g/g}$)	Plant height (cm)	Days to 50% tasseling (days)	Days to 50% silking (days)	Cob length (cm)	Number of kernel rows/cob	No of kernels/row	100 kernel weight (g)	Mean grain yield/plant (g)
1 ^a UMI1200 β^+ -1	9.073 \pm 0.557	126.50	58.00	61.00	13.10	12.00	10.00	24.10	87.12
2 ^a UMI1200 β^+ -2	8.303 \pm 0.364	126.10	59.00	59.00	13.40	10.00	10.00	24.10	87.32
3 ^a UMI1200 β^+ -3	7.056 \pm 0.562	126.80	57.00	61.00	13.20	12.00	09.00	24.20	87.61
4 ^a UMI1200 β^+ -4	7.251 \pm 0.453	126.30	58.00	60.00	13.50	12.00	10.00	24.60	88.12
5 ^a UMI1230 β^+ -1	9.232 \pm 0.508	133.90	57.00	59.00	12.10	10.00	09.00	23.89	76.41
6 ^a UMI1230 β^+ -2	9.021 \pm 0.471	134.10	56.00	58.00	12.30	10.00	08.00	23.78	76.39
7 ^b UMI1200 (RP)	0.893 \pm 0.070	127.00	60.00	62.00	13.70	14.00	11.00	25.00	88.21
8 ^b UMI1230 (RP)	1.610 \pm 0.083	135.00	58.00	60.00	12.50	12.00	10.00	24.10	76.71
9 ^b HP467-15 (DP)	10.525 \pm 0.263	110.00	56.00	59.00	13.00	10.00	10.00	23.00	54.80
10 Test inbred mean	8.322 \pm 0.485	128.95	57.50	59.67	12.93	11.00	9.33	24.11	83.83
11 Check inbred mean	4.342 \pm 0.138	124.00	58.00	60.33	13.07	12.00	10.33	24.03	73.24

^aTest inbreds and ^bCheck inbreds.

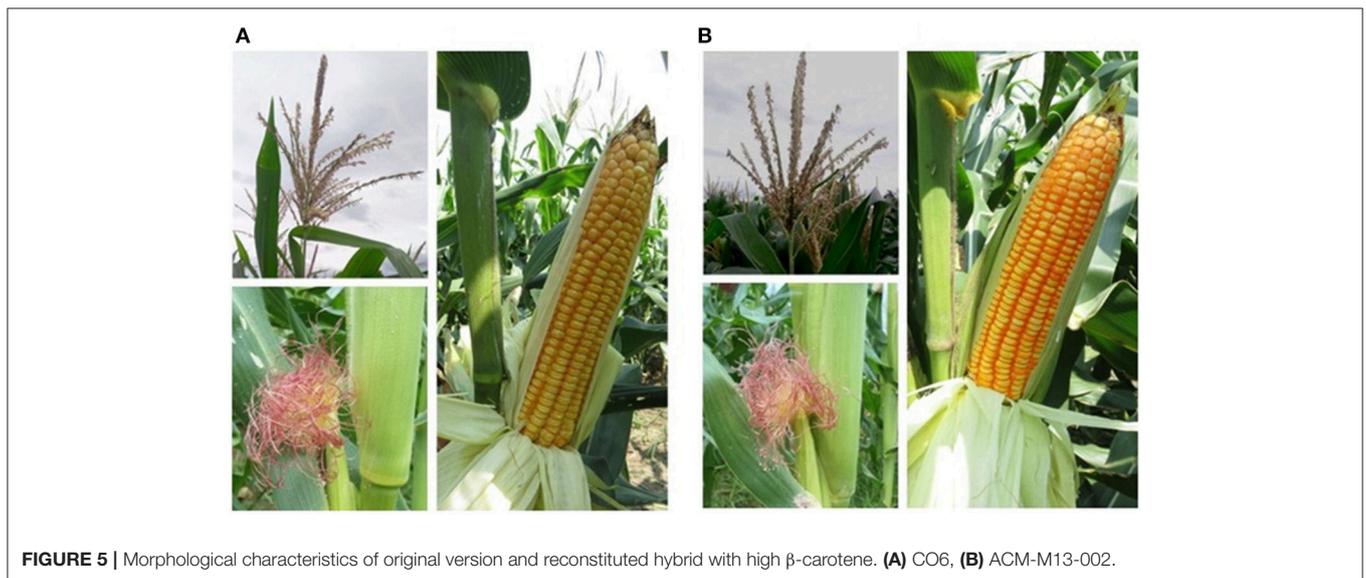


FIGURE 5 | Morphological characteristics of original version and reconstituted hybrid with high β -carotene. **(A)** CO6, **(B)** ACM-M13-002.

The β -carotene concentration of improved lines is presented in **Table 2**. In addition, the β -carotene concentration of the improved lines at different environments are summarized in **Supplementary Table 3**.

Evaluation of Hybrid Combination Under Comparative Yield Trial (CYT) and Multi-Location Trial (MLT)

Five hybrid combinations were developed using the improved lines. The agronomic performance and the β -carotene concentration of the five hybrids were evaluated along with their corresponding original hybrid (CO6) and a commercial hybrid (NK6240) under CYT (**Figure 5**). Among the five hybrids, ACM-M13-002 showed a 13.38% yield increase and a 7.3-fold β -carotene increase over its original hybrid and 15.59% yield increase over a commercial hybrid NK6240 (**Table 3**). Thus,

ACM-M13-002 forwarded to MLT along with eight hybrids to study the GE interaction over the different maize growing regions in Tamil Nadu. The yield performance of MLT showed that the hybrid ACM-M13-002 (G3) recorded a value that is comparable to that of its original hybrid (CO6) in all the locations. An average yield performance across 10 environments recorded 7,256.2 kg/ha, which was comparable with the original hybrid CO6 (G2) (7,358.1 kg/ha).

Per se performance of the yield for the developed five hybrids ranged from 10.73 to 11.86 (t/ha) with an average of 11.02 (t/ha). Analysis of variation for the grain yield (kg/ha) of maize hybrids showed a significant difference between the components under study (Genotypes, Environments and Genotype \times Environment) with the percent accounting for the highest total variation sum of the square by 80% for environments, 1.33%, and 18.68% for GE (**Tables 4, 5** and **Supplementary Table 4**). The effect of the genotype and environment is 14.04 times higher compared

TABLE 3 | Agronomic performance and β-carotene value of hybrids developed through MABB under comparative yield trial (CYT).

Hybrid	Plant height (cm)			Days to 50% tasseling			Days to 50% silking			1,000 grain weight (g)			Grain yield/plant (g)			Yield (t/ha)	β-carotene (μg/g)
	2014–15		2015	2014–15		2015	2014–15		2015	2014–15		2015	2014–15		2015		
	VGD ¹ Rabi	VGI ² Rabi	VGD ¹ Kharif	VGI ² Rabi	VGD ¹ Rabi	VGI ² Rabi	VGD ¹ Rabi	VGI ² Rabi	VGD ¹ Rabi	VGI ² Rabi	VGD ¹ Rabi	VGI ² Rabi	VGD ¹ Rabi	VGI ² Rabi	% increase over CO6		
^a ACM-M13-001 ³	185.00	184.00	186.00	46.00	47.00	48.00	49.00	49.00	343.00	344.00	163.00	164.00	164.00	164.00	4.49	6.53	8.92
^a ACM-M13-002 ³	187.00	186.00	188.00	45.00	46.00	48.00	48.00	48.00	329.00	332.00	179.00	176.00	178.00	178.00	13.38	15.59	9.05
^a ACM-M13-003 ³	238.00	237.00	239.00	49.00	49.00	53.00	52.00	52.00	401.00	400.00	160.00	162.00	162.00	162.00	2.58	4.58	8.24
^a ACM-M13-004 ³	206.00	205.00	206.00	48.00	49.00	51.00	52.00	52.00	420.00	418.00	164.00	162.00	163.00	163.00	3.82	5.84	8.47
^a ACM-M13-005 ³	208.00	207.00	208.00	48.00	49.00	52.00	52.00	52.00	339.00	341.00	161.00	162.00	160.00	160.00	2.58	4.58	8.83
SE ⁴	9.55	9.55	9.53	0.73	0.63	1.03	0.87	0.86	18.39	17.49	3.47	2.73	3.22	2.04	2.08	2.08	0.15
SD ⁵	21.35	21.35	21.30	1.64	1.41	2.30	1.95	1.92	41.13	39.12	7.77	6.10	7.20	4.55	4.64	4.64	0.33
^b CO6 ⁶	189.00	188.00	190.00	53.00	53.00	56.00	56.00	55.00	401.00	400.00	157.00	159.00	157.00	157.00			1.24
^b NK6240 ⁶	200.00	198.00	200.00	49.00	50.00	52.00	53.00	51.00	331.00	330.00	168.00	169.00	167.00	167.00			0.71
Test hybrid mean	204.80	203.80	205.40	47.60	48.00	50.40	50.60	49.80	366.40	367.00	165.40	165.20	165.40	165.40	5.37	7.42	8.7
Check hybrid mean	194.50	193.00	195.00	51.00	51.50	54.00	54.50	53.00	366.00	365.00	162.50	164.00	162.00	162.00			0.97

¹Vaigai dam, ²Vagarai, ³High beta carotene hybrids, ⁴Standard error, ⁵Standard deviation, ⁶Standard checks. ^aTest hybrids, ^bCheck hybrids. Note: Yield (t/ha) given is the average of the hybrids.

to the component genotype. Hence, the variation that exists among the genotypes across the environments paves the way to understanding the yield stability of the hybrids over the varying environments and specific adaption of the hybrids. Based on the Interaction Principal Component Axis 1 (IPCA1) of the AMMI model, the genotypes G1, G2, and G5 recorded values nearer to the zero on the biplot (0.056, -0.154, and 0.002) which represents that the genotypes are highly stable with good yield potential (Figure 6). From the “Which won where” polygon view of GGE analysis (Figure 7) four genotypes - G3, G4, G5, and G8 - were located at the corner, which denotes that the genotypes are specifically adapted to the particular environment for the trait under study. Average Environment Coordination (AEC) recorded that G3 is placed at the second concentric circle and is ideal in terms of high yielding ability and stability, whereas other genotypes, G2, G6, and G7, placed at the third and fourth concentric circle where the stability gradient is low compared to the genotypes closer to the center axis. Based on the vertices of the Average Environment Axis (AEA), the environments E9 and E4 were considered as highly interactive environments from where the wider adaption of the genotypes are selected.

DISCUSSION

CO6 is one of the popular maize hybrids of India, especially in Tamil Nadu. CO6 is known for its high yield, multiple disease resistances, and its suitability for rainfed and irrigated conditions. However, CO6 is low in pVAC β-carotene, thus, the present investigation was taken up to improve the β-carotene in CO6 through MABB. Previously, Muthusamy et al. (9) and Zunjare et al. (10) used an accelerated MABB scheme to generate the improved version of VQL1-K10-40-11-53 × VQL2-K10-08-14, HQPM7-B, HQPM 5-C hybrids for β-carotene. We also used the same MABB scheme to enrich the β-carotene in CO6. Marker-assisted foreground selection using gene-linked markers permits the transfer of the gene of interest with high precision in MABB. The *crtRB1* gene-specific marker (*crtRB1* 3’TE) used in this study allows us to detect the positive plants exactly without any false positives at any stage of MABB. The advantages of using a linked marker for the selection of single or multiple genes simultaneously in MABB have been described in many studies for nutritional traits in maize (7, 8, 13). The backcross and selfed-generations from both crosses, UMI1200 × HP467-15 and UMI1230 × HP467-15, showed segregation distortion. The segregation pattern of allele 1 and allele 3 deviated from the expected Mendelian ratio (1:1 and 1:2:1) and allele 1 was diminished. These results are similar to the reports of Babu et al. (8), who described the segregation distortion of allele 1 in five out of eight populations. There are many facts, such as embryo-specific mutation (24), segregation distortion regions in the maize genome (25), mutants like the defective kernel (24), gametophytic factors (24, 26), and genetic background of the target allele (8), that are reported to be the reason for the segregation distortion. In this case, the evaluation of a large population for attaining enough foreground positives is essential. Marker-assisted background selection using SSR markers helped

TABLE 4 | Analysis of variance of yield data of nine maize hybrids tested across environments during Kharif 2016 and 2017.

S.No	Source	DF	SS	MS	Total variation explained (%)
1	Environment (E)	9	391935207	43548356**	80.00
2	Genotype (G)	8	6505899	813237**	1.33
3	Genotype × Environment Interaction (GE)	72	91495843	1270776**	18.68
4	PC1	16	54822906	3426432**	59.76
5	PC2	14	15431914	1102280**	16.82
6	PC3	12	8193433	682786**	8.93
7	Residuals	90	2241107	24901**	0.00

**Represents highly significant at 1% level.

TABLE 5 | Multi location trial (MLT) conducted in Kharif 2016 and 2017.

S. No Hybrid	Parents	Code for hybrids	Grain yield (Kg/ha) kharif 2016				Grain yield (Kg/ha) kharif 2017							
			CBE ^{E1}	VGI ^{E2}	VRI ^{E3}	BSR ^{E4}	CBE ^{E5}	VGI ^{E6}	VRI ^{E7}	BSR ^{E8}	VGDE ^{E9}	CEM ^{E10}	Mean	
1	NK6240	-	G1	8,705.0	8,598.5*	5,508.0	6,564.5	10,447.5	5,023.5	8,220.5	6,141.5	5,671.5	8,347.0	7,322.8
2	CO6	UMI1200 × UMI1230	G2	8,796.5	9,466.5	5,912.0	6,008.5	10,125.0	6,12.0	8,381.5	5,554.0	5,794.0*	7,531.0	7,358.1
3	ACM-M13-002	UMI1200β ⁺ × UMI1230β ⁺	G3	8,794.5	8,824.0	6,875.5*	7,138.5*	8,309.0	6,452.5	7,615.5	6,393.5*	6,393.5*	5,765.0	7,256.2
4	ACM-M13-007	UMI285pa × UMI1200-1	G4	8,477.5	8,474.5	6,681.5*	6,493.5	10,525.0	5,715.0	9,499.0*	5,225.5	6,005.5*	4,693.5	7,179.1
5	CMH 11-583	N09-153-3A × N10-65-3	G5	8,984.5*	8,264.0	5,918.0	5,563.0	10,349.5	5,478.5	8,892.0*	5,745.0	4,730.0	7,622.0	7,154.7
6	VaMH 14020	UMI1230 × VIM153	G6	8,147.0	8,850.0	6,882.5*	6,557.0	10,258.0	6,002.0*	9,008.0*	5,702.0	5,837.5*	9,664.0*	7,690.8
7	VaMH12014	UMI1200 × VIM357	G7	7,507.0	8,574.5	6,324.0	6,695.0*	9,766.5	6,070.5*	8,583.5	6,041.5	5,305.0	9,876.0*	7,474.4
8	900 M GOLD	-	G8	8,989.0*	8,127.5	5,110.0	6,255.0	9,249.5	6,118.0*	8,913.5*	6,393.5*	5,094.0	9,263.5*	7,351.4
9	CMH 11-586	N09-164-2 × N148	G9	9,435.5	8,319.5	7,333.5*	6,416.5	10,618.0*	5,550.5	8,235.5	5,978.5	5,237.5	9,924.0*	7,704.9
10	^a SEd			140.37	115.25	87.36	116.95	257.63	65.31	110.11	133.76	82.26	138.49	
11	^b CD (p = 0.05)			323.71	265.76	201.46	269.68	594.11	150.60	253.92	308.45	189.70	319.37	
12	Mean			8,648.5	8,611.0	6,282.8	6,410.8	9,960.9	5,824.7	8,594.3	5,908.3	5,563.8	8,076.2	

(E1) Coimbatore, (E2) Vagarai, (E3) Vridhachalam, (E4) Bhavanisagar, (E5) Coimbatore, (E6) Vagarai, (E7) Vridhachalam, (E8) Bhavanisagar, (E9) Vaigai dam, and (E10) Athiyandal, ^aStandard error deviation, ^bCritical difference. *Represents significant at 5% level.

in selecting the foreground positive plants with a high RPGR. We performed the background selection starting from the BC₁F₁ generation; it hastened the RPGR in advanced lines. Following this approach, after only two generations of backcrossing, it was possible to identify the plants carrying RPGR ranging from 90.24 to 92.42% (UMI1200 × HP467-15) and 90.41–92.21% (UMI 1230 × HP467-15) in BC₂F₃ improved lines. These results are in accordance with the previous studies (9, 27, 28).

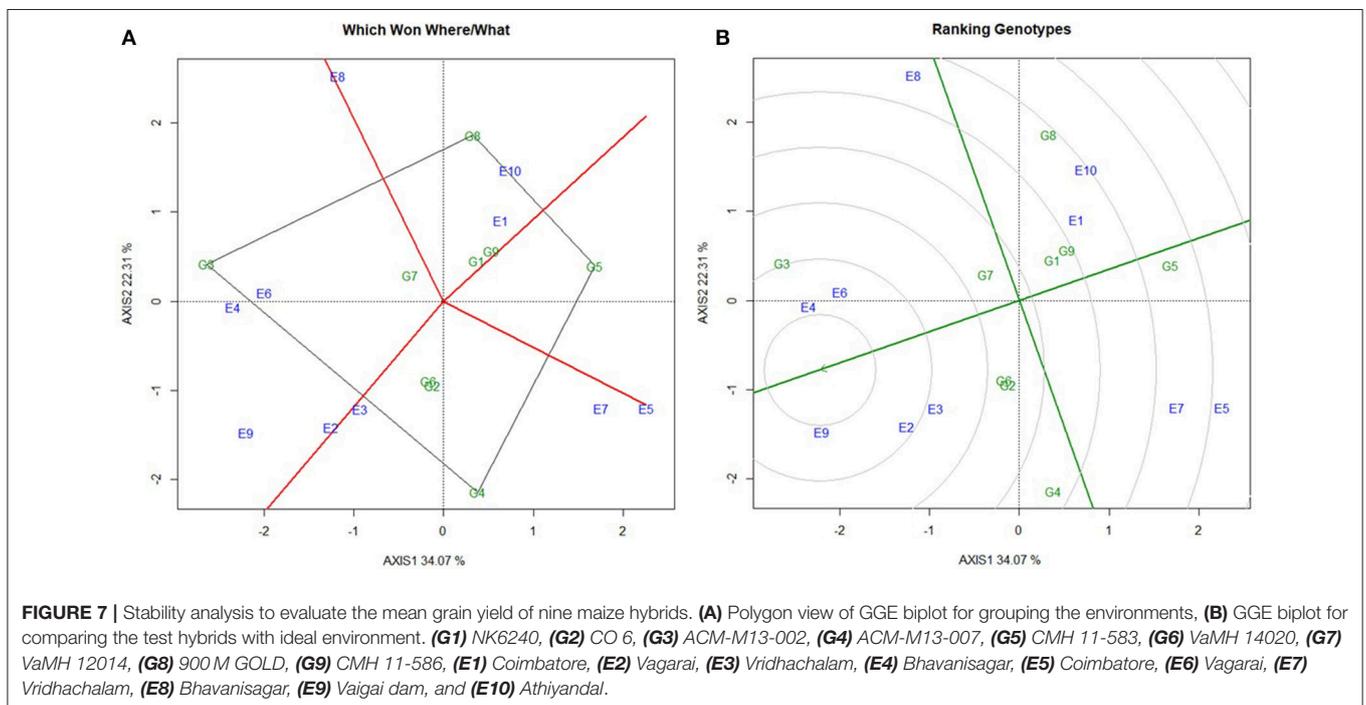
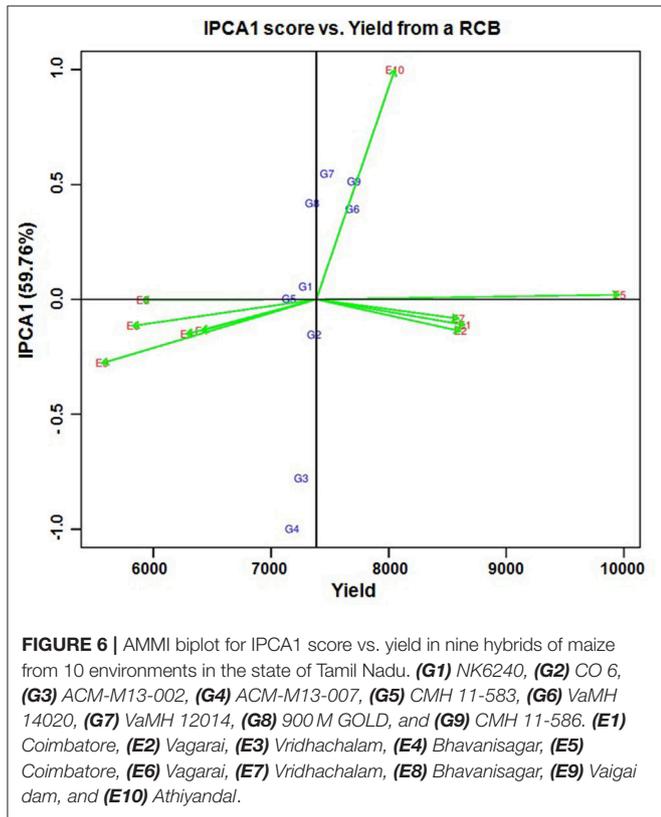
The study aims to introgress the *crtRB1* gene without troubling the recurrent parental genome by MABB, thus the improved line will be suitable for further use. For confirming the suitability of the new genotypes, it is essential to characterize and choose the progenies that are closer to the parent for both morphological and nutritional traits as this selection adds value to the MABB program. Thus, we investigated the agro-morphological characters of the improved lines. It revealed that the agro-morphological characters of the improved lines were on par with the recipient parent. The agro morphological characters among the six improved lines ranged from 71.43 to 99.89%. Among them, UMI1200β⁺-1, UMI1200β⁺-2, and UMI1200β⁺-4 from UMI1200 × HP467-15, and UMI1230β⁺-1 from

UMI1230 × HP467-15 recorded more than 90% recovery for the traits *viz.*, days to tasseling, plant height, cob length, number of kernels per row, 100 kernel weight, and grain yield per plant. Moreover, the β-carotene content of the improved lines ranged from 7.056 to 9.073 μg/g (UMI1200 × HP467-15) and 9.021–9.232 μg/g (UMI1230 × HP467-15). The β-carotene concentration of the improved lines is on par with the β-carotene parent. Similar results were obtained in other studies (10, 29).

The improved lines can be considered as the candidate parents for developing β-carotene hybrids adapted to irrigated and rainfed conditions. They were successfully used to develop the five hybrid combinations. Among them, ACM-M13-002 is a superior hybrid with increased yield and β-carotene concentration over its original hybrid and a commercial hybrid NK6240 under CYT. Hence, this hybrid was forwarded for MLT along with eight hybrids to study the GE interaction over the different maize growing regions in Tamil Nadu. The yield performance of MLT showed that ACM-M13-002 (G3) recorded a value that is comparable to that of its original hybrid CO6 (G2) in all the locations. Based on the stability ANOVA, the GE toward the yield was lesser compared to the environment

(E) and it is 14.04 times higher than that of genotype (G). These results are similar to the earlier study on maize (20). We observed significant genotype by environment interaction (GE)

effect, which revealed that the yield performance of all the hybrids over the environments under study was not consistent. This situation necessitates understanding the nature and magnitude of genotype by environment effect which may not be possible through standard ANOVA (21, 22). IPCA1 from the AMMI model gives an idea about the association of the genotypes with the environments under study. A higher IPCA1 value reflects the specific adaptation of the genotypes irrespective of the direction in which it falls (Positive/ Negative) and the values nearer to the zero reflect the genotypes widely adapted to all the environments tested. From the above concept the genotype G1, G2, and G5 recorded values closer to the zero, which could be promising genotypes for the yield trait over all the environments. GGE biplot analysis reflects the similarity existing between the genotypes, environment, and their corresponding response to each other, as well as the interaction between them, making it the best tool to graphically represent the stability of the genotype (23). From the polygon view of GGE biplot, the genotypes G3, G4, G5, and G8 are recorded as a specific adaptation to the respective environments which are present nearer to the vertex of the polygon. High *per se* performance, higher vector length, and high stability across the environments are the properties of ideal genotype and the genotypes places closed to the ideal genotype are called desirable genotypes. These are most probably at the second concentric circle of the GGE biplot (23), with the greatest ability to discriminate genotypes, favoring the selection of superior genotypes. The GGE biplot revealed that the genotype G3 (ACM-M13-002) could be recommended for yield trait to all the environments studied since it is placed nearer to the highly interactive environment (E4) and also at the second concentric circle on the GGE biplot.



In summary, we have successfully improved the β -carotene concentration in the CO6 hybrid through MABB. The β -carotene enriched hybrid developed in this study will hold great promise for nutritional and food security. Also, the improved maize inbreds with β -carotene will serve as a potential genetic material for the development of β -carotene rich cultivars in maize breeding programs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

SN conceived and designed the methods and experiments. SN, TD, GK, NT, and RR constructed backcross progenies and managed field work. TD, BP, SC, JN, DM, KA, and VS conducted phenotype, genotype, and biochemical analyses. SN, BP, SC, and DM analyzed the data. LM and SM provided suggestions on experiments and data analysis. SN, KA, DM, and SC drafted the

manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnut.2020.00134/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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