Development of Biofortified Maize Hybrids through Marker-Assisted Stacking of $\beta$-Carotene Hydroxylase, Lycopene-$\varepsilon$-Cyclase and Opaque2 Genes

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Traditional yellow maize though contains high kernel carotenoids, the concentration of provitamin A (proA) is quite low (<2 µg/g), compared to recommended level (15 µg/g). It also possesses poor endosperm protein quality due to low concentration of lysine and tryptophan. Natural variant of $crtRB1$ ($\beta$-carotene hydroxylase) and $lcyE$ (lycopene-$\varepsilon$-cyclase) cause significant enhancement of proA concentration, while recessive allele, opaque2 (o2) enhances the level of these amino acids. Development of biofortified maize enriched in proA, lysine and tryptophan thus holds significance in alleviation of micronutrient malnutrition. In the present study, marker-assisted stacking of $crtRB1$, $lcyE$ and o2 was undertaken in the genetic background of four maize hybrids (HQPM1, HQPM4, HQPM5, and HQPM7) popularly grown in India. HP704-22 and HP704-23 were used as donors, while four elite QPM parents viz., HKI161, HKI163, HKI193-1, and HKI193-2 were used as recipients. $CrtRB1$ showed severe segregation distortion, while $lcyE$ segregated as per the expectation. Recovery of recurrent parent genome (RPG) among selected backcross progenies ranged from 89 to 93%. Introgressed progenies possessed high concentration of proA (7.38–13.59 µg/g), compared to 1.65–2.04 µg/g in the recurrent parents. The reconstituted hybrids showed an average of 4.5-fold increase in proA with a range of 9.25–12.88 µg/g, compared to original hybrids (2.14–2.48 µg/g). Similar plant-, ear-, and grain- characteristics of improved versions of both inbreds and hybrids were observed when evaluated with their respective original versions. Mean lysine (0.334%) and tryptophan (0.080%) of the improved hybrids were at par with the original versions (lysine: 0.340%, tryptophan: 0.083%). Improved hybrids also possessed similar grain yield potential (6,301–8,545 kg/ha) with their original versions (6,135–8,479 kg/ha) evaluated at two locations. This is the first study of staking $crtRB1$-, $lcyE$-, and o2-, favorable alleles in single genetic background. The improved
INTRODUCTION

Micronutrient malnutrition popularly known as "hidden hunger" is a serious health problem worldwide, particularly in the under-developed and developing countries (Bouis and Saltzman, 2017). Nearly two billion people suffer from deficiency of micronutrients, while 815 million people are under-nourished (Global Nutrition Report, 2017). Among micronutrients, vitamin A plays a key role in human metabolism. This deficiency leads to visual blindness which may cause eye sight damage to millions of preschool-age children. According to HarvestPlus, nearly 20 million pregnant women are vitamin A deficient, while out of which about one-third are clinically night-blind. There are about one-half of these cases occur in India with severe form of vision impairment. The deficiency of lysine and tryptophan leads to fatigue, delayed growth, loss of appetite, depression, anxiety in children (Nuss and Tanumihardjo, 2010; Jompuk et al., 2011). Moreover, unbalanced protein in the diet leads to protein energy malnutrition (PEM) that affects more than a billion people across the world (Bain et al., 2013). The adoption of quality protein maize (QPM) varieties possessing balanced protein due to higher lysine and tryptophan which has shown significant promise in solving problem of PEM across the world (Nyakurwa et al., 2017).

Cereals are rich source of energy, but lacking the required content of micronutrients (Nuss and Tanumihardjo, 2010). Genetic enhancement of micronutrient in crops through plant breeding known as “biofortification” which is a cost-effective and sustainable process, where micronutrients reach the target group in their natural form (Pfeiffer and McClafferty, 2007; Gupta et al., 2015; Neeraja et al., 2017). Maize occupies an important position in the world economy. It along with rice and wheat provides at least 30% of the food calories to more than 4.5 billion people in 94 developing countries, besides serving as a major component of animal feed (Shiferaw et al., 2011). In India, maize is the third most cereal after rice and wheat, and used as an important source of both food and feed (Yadav et al., 2015). Normal maize protein contains lower level of lysine (0.16–0.26%) and tryptophan (0.02–0.06%) which is less than half of the recommended dose specified for human nutrition (Bjarnason and Vasal, 1992; Vivek et al., 2008). Further, traditional yellow maize contains enough kernel carotenoids as compared to other cereals. However, it is predominated by non-proA fractions and contains only 0.25–2.50 µg/g of proA carotenoids which is far below the nutritional requirement (15 µg/g) for humans (Pixley et al., 2013).

Favorable alleles of lycopene e-cyclase (lcyE) and β-carotene hydroxylase1 (crtRB1) genes causes enhancement in proA in maize (Harjes et al., 2008; Yan et al., 2010; Babu et al., 2013). The recessive opaque2 (o2) allele enhances endosperm lysine and tryptophan by almost 2-folds (Mertz et al., 1964). Marker-assisted selection (MAS) using very low expensive DNA markers helps in stacking of multiple target genes into a genetic background without progeny testing (Das et al., 2017). It also significantly reduces the breeding cycles required to reconstitute the recurrent parent genome (RPG) (Gupta et al., 2013). Further, high cost of HPLC (High Performance Liquid Chromatography) analyses for estimation of micronutrients among individuals of segregating populations could be avoided through usage of molecular markers. The successful examples of application MAS in development of nutritious maize hybrids in India have been the commercial release of “Vivek QPM9” (Gupta et al., 2013), “Pusa Vivek QPM9 Improved” (Muthusamy et al., 2014), “Pusa HM4 Improved,” “Pusa HM8 Improved,” and “Pusa HM9 Improved” (Hossain et al., 2017). Lysine and tryptophan rich QPM hybrids of late maturity so far released in the India do not contain recommended level of proA concentration. The present study was thus aimed to (i) stack favorable alleles of crtRB1, lcyE and opaque2 genes into elite inbreds/hybrids by using marker-assisted backcross breeding (MABB) and (ii) evaluate the MABB-derived –inbreds/hybrids for nutritional quality, agronomic and yield related traits.

MATERIALS AND METHODS

Plant Materials

The parental inbreds viz., HKI161, HKI163, HKI193-1, and HKI193-2 of four QPM hybrids, [HQPM1 (HKI193-1 × HKI163), HQPM4 (HKI193-2 × HKI161), HQPM5 (HKI163 × HKI161) and HQPM7 (HKI193-1 × HKI161)], were targeted for enrichment of micronutrients. The popular and commercial maize hybrids are adapted to diverse agro-ecologies of India (Table 1). Recurrent parents were crossed with donor lines and four crosses viz., cross-I (HKI161 × HP704-23), cross-II (HKI163 × HP704-22), cross-III (HKI193-1 × HP704-23), cross-IV (HKI193-2 × HP704-22) were attempted to stack crtRB1, lcyE, and o2 in the genetic background of recurrent parents. The pedigree information of the recurrent parents and donors is given in Table S1.

Generation of Backcross-and Self-progenies

Backcross- and self- generations which were grown at different locations are described in Table S2, and the MABB scheme followed is represented as Figure 1. The recipients and donors showing polymorphism for gene-based markers were crossed during rainy season (July-November 2012) at IARI, New Delhi (28°089N, 77°129E, 229 MSL). Hybridity of the F1's was tested using gene-based markers, and the true F1's were

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TABLE 1 | Details of popular commercial QPM hybrids targeted for provitamin A enrichment.

| S. No. | Hybrid   | Parental lines       | Maturity group | Maturity (days) | Year of release | Area of adaptation |
|--------|----------|----------------------|----------------|----------------|-----------------|-------------------|
| 1.     | HQPM1    | HK0193-1 × HK163     | Late           | 88-90          | 2005            | Zone-II, III, IV, V |
| 2.     | HQPM4    | HK0193-2 × HK0161    | Late           | 95-97          | 2010            | Zone-II, III, IV, V |
| 3.     | HQPM5    | HK0163 × HK0161      | Late           | 92-93          | 2007            | Zone-II, III, IV, V |
| 4.     | HQPM7    | HK0193-1 × HK161     | Late           | 96-97          | 2008            | Zone-IV           |

Zone II, North Western Plain Zone; Zone III, North Eastern Plain Zone; Zone IV, Peninsular Zone; Zone V, Central Western Zone.

FIGURE 1 | Marker-assisted backcross breeding scheme followed for development of provitamin A, lysine and tryptophan rich maize hybrid (e.g., HQPM7). RP, Recurrent Parent; DP, Donor Parent.

backcrossed to their corresponding recurrent parent during winter season (December, 2012-April, 2013) at Winter Nursery Centre (WNC), Hyderabad (17.2°198N, 78.1°249E, 542.6 MSL). The BC1F1 progenies were grown at IARI, New Delhi during rainy season (2013), and foreground selection was carried out (Figure S1). The foreground positive plants with high recovery of RPG (RPG) and maximum phenotypic similarity were further backcrossed to the recurrent parent. The BC2F1 progenies were grown at WNC, Hyderabad during winter season (2013-14), and were subjected to foreground-, background- and phenotypic selection. The BC2F2 progenies were raised during rainy season (2014) at IARI, New Delhi. Foreground positive plants homozygous for all genes were subjected to background- and phenotypic- selection. The selected plants were subsequently self-pollinated to generate BC2F3 and BC2F4 progenies (Table S2).

Marker-Assisted Foreground Selection

Three SSR markers based on o2 gene, viz., phi057, phi112, and umc1066 were screened to distinguish the parental lines, of which, phi057 marker revealed polymorphic pattern between recipients and donors (Gupta et al., 2013). Polymerase chain reaction (PCR) amplification for SSRs was followed as per Hossain et al. (2017). Four percent of Seakem LE agarose (Lonza, Rockland, ME USA) gel was used for electrophoretic separation of PCR products at 120 V for 3–5 h along with 100 bp DNA ladder (MBA-Fermentas). Gene-based InDel marker present in 3’TETE region of crcRB1 and lcyE were used for foreground selection (Harjes et al., 2008; Yan et al., 2010; Figure S1; Table 2). PCR mediated amplification of crcRB1 and lcyE was performed using protocol standardized at Maize Genetics Unit, IARI (Zunjare et al., 2017a). Agarose of 1.5% concentration (Lonza, Rockland, ME USA) was used for separating the amplicon at 120 V for 2–3 h along with 100 bp DNA ladder (MBA-Fermentas). The amplified products were visualized using a gel documentation system (Alpha Innotech, California, USA) and scored for the presence and absence of designated allele.

Marker-Assisted Background Selection

SSRs with near uniform coverage across 10 chromosomes of maize genome were used for polymorphism survey between the respective recurrent and donor genotypes (Table 3, Table S3).
The primer sequences of the SSRs were retrieved from the maize genome database (www.maizegdb.org) and were custom synthesized (Sigma Tech., USA). PCR amplification and scoring of amlicons was undertaken as per Hossain et al. (2017). The markers which were polymorphic between the recurrent and their respective donor parents were employed for recovering the RPG in individuals of BC$_1$F$_1$, BC$_2$F$_1$, and BC$_2$F$_2$ generations.

**Phenotypic Selection**
Selection of plant-, ear-, and grain-characteristics was performed among the individuals of each backcross- and self- generations for their similarity with their respective recurrent parents. The harvested BC$_2$F$_3$ seeds from the introgressed progenies were subjected to standard light box test along with the original recurrent parental seeds to measure the intensity of opaqueness (Hossain et al., 2008). The seeds with similar degree of opaqueness of the original inbreds were forwarded for further generation and the reconstitution of hybrids (Vivek et al., 2008; Gupta et al., 2013).

**Analysis of Provitamin A, Lysine, and Tryptophan**
The selfed seeds of BC$_2$F$_4$ plants (two BC$_2$F$_3$ populations for HK1163) were utilized for biochemical analysis. The selfed ears were harvested at moisture level 12–14%, and then cleaned and dried under the shade. The equal amount of grains shelled from ears of same families was bulked together, and the samples thus drawn were stored in ambient temperature (22–26°C) for 2 months before biochemical analysis.

The extraction of β-carotene (BC) and β-cryptoxanthin (BCX) from maize seeds was carried out using procedures described by Kurlich and Juvik (1999) and Vignesh et al. (2012). Quantification of the BC and BCX was carried out with a Dionex Ultimate 3000 UHPLC system (Ultra High Performance Liquid Chromatography; Thermo Scientific, Massachusetts, USA). Samples were eluted through Acclaim™ 120 C$_{18}$ column (5 µm, 120A°, 4.6 × 150 mm, Thermo Scientific) and detected with a RS photodiode array detector (PDA) with absorbance in 265 and 280 nm wavelength, respectively for lysine and tryptophan. Final concentration of the amino acids in each sample was estimated by standard regression using external standards (AAS 18-5 ML, Sigma Aldrich).

The protocol standardized by Sarika et al. (2017) was followed to estimate lysine and tryptophan content of maize endosperm. Amino acids were estimated by using Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Massachusetts, USA). The samples were eluted through Acclaim™ 120 C$_{18}$ column (5 µm, 120A°, 4.6 × 150 mm, Thermo Scientific) and detected with a RS photodiode array detector (PDA) with absorbance in 265 and 280 nm wavelength, respectively for lysine and tryptophan. Final concentration of the amino acids in each sample was estimated by standard regression using external standards (AAS 18-5 ML, Sigma Aldrich).

**Evaluation of Introgressed Inbreds**
Twelve improved progenies (BC$_2$F$_3$/BC$_2$F$_4$) along with the respective recurrent parents were evaluated during rainy season (2015) at IARI Experimental Farm, New Delhi. Two-three plants per entry were self-pollinated, and selfed grains were analyzed for proA, lysine and tryptophan. Characters viz., days to 50% male flowering (MF), days to 50% female flowering (FF), plant height (PH), ear height (EH), ear length (EL), ear width (EW), number of rows (NR), number of kernels per row (NKR), and 100-seed weight (TW) were recorded from open pollinated plants.

**Evaluation of Reconstitution of Hybrids**
Selected 12 (BC$_2$F$_3$/BC$_2$F$_4$) progenies of the four improved inbreds were used to reconstitute twelve F$_3$ hybrids during winter season (2015-16) at WNC, IIMR, Hyderabad. Three versions of the reconstituted hybrids (-A, -B, and -C) and their corresponding original hybrids were evaluated in Randomized Complete Block Design (RCBD) with two replications at two diverse maize growing zones of the country viz., IARI Experimental Farm, New Delhi in Northern India and IARI Regional Research Centre, Dharwad (15°21’9N, 75°05’9E, 750 MSL) Karnataka in Southern India during rainy season of 2016. Two to three plants in each of the hybrid entries were self-pollinated. Since, proA (Vignesh et al., 2012), lysine and tryptophan (Pixley and Bjarnason, 2002) do not vary much across locations, selfed seeds from IARI Experimental Farm, New Delhi were used for analysis of quality traits. However, morphological traits viz., MF, FF, PH, EH, EL, EW, NR, NKR, TW, and grain yield (GY) were recorded in open pollinated plants at both the locations.

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**TABLE 2 |** Details of gene-based markers used for foreground selection in MABB.

| S. No. | Gene | Bin location | Marker | Primer Sequence (5′–3′) | Primer | References |
|-------|------|--------------|--------|--------------------------|--------|------------|
| 1.    | crtRB1 | 10.05        | 3′TE InDel | ACACACATGGACAAATTCGG | Forward | Yan et al., 2010 |
|       |       |              |        | ACACTCTGCGCCATGAACAC | Reverse 1 |              |
|       |       |              |        | ACAGCTAAGGGGAGCCAG | Reverse 2 |              |
| 2.    | lcyE  | 8.05         | 5′TE InDel | AAGGAGGAGGAGCAGACAC | Forward | Babu et al., 2013 |
|       |       |              |        | GAGGGAGGAGCAGAGACAC | Reverse |              |
| 3.    | opaque2 | 7.01        | phi057 | CTATCAAGTGGGCTGTCCAT | Forward | Gupta et al., 2013 |
|       |       |              |        | CAGTCGGAAAGAACCCTGGC | Reverse |              |
Statistical Analysis

The observed segregation pattern of *crtRB1* and *lcyE* across segregating populations (BC1F1, BC2F1, and BC2F2), and o2 in BC1F1 generation of four crosses was tested for goodness of fit by $\chi^2$ analysis. The amplicons of markers used in background selection were scored as "A" for the recipient allele, "B" for the donor allele, and "H" for the heterozygous genotype. Recovery percentage of RPG was estimated using formula, RPG (%) = $\frac{[A + (0.5H)/(A + B + H)] \times 100$ (Benchimol et al., 2005). The recovery of RPG among selected backcross-derived progenies was also established using Graphical Geno Types (GGT) version 3.0 (Van-Berloo, 1999). Graphical representation based on mean of improved proA, lysine and tryptophan was ascertained by Microsoft Excel (2013). Agronomic and biochemical data of hybrids were analyzed using Windostat 8.5 software package (Khetan, 2005).

RESULTS

Marker- and Trait-Polymorphism among Parents

All the four recurrent parents (HKI161, HKI163, HKI193-1, and HKI193-2) revealed unfavorable allele (C+: 296 bp), while the donors possessed favorable allele (C: 543 bp) of *crtRB1* gene. Polymorphism test for *lcyE* revealed the presence of favorable allele (L: 650 bp) in two recurrent (HKI161 and HKI163) and two donor (HP704-22 and HP704-23) parents, while HKI193-1 and HKI193-2 possessed unfavorable allele (L+: 300 bp). All the recurrent parents low proA concentration (HKI161: 2.04 $\mu$g/g, HKI163: 1.65 $\mu$g/g, HKI193-1: 1.84 $\mu$g/g, and HKI193-2: 1.74 $\mu$g/g), while donor parents possessed high proA concentration (HP704-22: 16.05 $\mu$g/g and HP704-23: 15.28 $\mu$g/g). Based on phi057, recessive allele of o2 (165 bp) was present in all QPM recurrent parents while, the donors possessed dominant allele, O2 (159 bp). The lysine (HKI161: 0.308%, HKI163: 0.347%, HKI193-1: 0.323%, and HKI193-2: 304%) and tryptophan (HKI161: 0.076%, HKI163: 0.082%, HKI193-1: 0.078%, and HKI193-2: 0.071%) content of recurrent parents was higher than their donor parents (Lysine, HP704-22: 0.176% and HP704-23: 0.192%; Tryptophan, HP704-22: 0.028% and HP704-23: 0.035%). A total of 114, 127, 133, and 124 polymorphic SSRs with polymorphism of 54.81, 61.06, 63.94, and 59.62% were observed in HKI161 × HP704-23, HKI163 × HP704-22, HKI193-1 × HP704-23, and HKI193-2 × HP704-22, respectively (Table 3, Table S3). The number of polymorphic markers in each chromosome ranged 7–17. These polymorphic markers were used for background selection for recovering the RPG in the backcross-derived populations.

Marker-Assisted Stacking of *crtRB1* *lcyE* and o2

The hybridity test in F1 generation has confirmed the success of crossing of parental lines. In BC1F1 and BC2F1 populations, the range of 100–120 and 106–122 plants, respectively were subjected to foreground selection of *crtRB1* gene (Figure S1). The heterozygous progenies for *crtRB1* were then subjected to foreground selection of *lcyE*. The polymorphic pattern for *lcyE* was observed only for viz., HKI193-1 × HP704-23 and HKI193-2 × HP704-22. The progenies (C+C/L+ in cross-I and -II, and C+C/L-L in cross-III and -IV) were further subjected to foreground selection for o2 allele using phi057. The progenies with o2 allele in homozygous state were selected in BC1F1. The population size used for analysis, segregation pattern, chi-square test results are mentioned in Table 4. Subsequently, foreground positive plants were analyzed for background selection using polymorphic markers. The recovery of RPG varied from 70.47 to 80.83% across four BC1F1, while RPG varied from 83.07 to 90.60% in the four BC2F1 were observed (Table 5). Stringent phenotypic selection was also applied considering plant architecture, ear- and grain-related traits. Foreground selection was executed among the plants in cross-I, -III and -IV, respectively to identify plants of genotype CC/LL/o2o2 (Table 3). For cross-II, two BC2F2 populations, BC2F2-I and BC2F2-II were raised in rainy season.

### Table 3: Number of screened SSRs and percentage polymorphism observed in four crosses.

| LG  | No. of markers screened | HKI161 × HP704-23 |  | HKI193-1 × HP704-23 |  | HKI193-2 × HP704-22 |  |
|-----|------------------------|--------------------|---|---------------------|---|--------------------|---|
|     |                        | NP Pol. (%)        |   | NP Pol. (%)         |   | NP Pol. (%)        |   |
| 1   | 17                     | 11 64.71           |   | 13 76.47            |   | 11 64.71           |   |
| 2   | 21                     | 10 47.62           |   | 12 57.14            |   | 12 57.14           |   |
| 3   | 29                     | 17 58.62           |   | 12 41.38            |   | 18 62.07           |   |
| 4   | 23                     | 11 47.83           |   | 14 60.87            |   | 14 60.87           |   |
| 5   | 21                     | 13 61.90           |   | 14 66.67            |   | 15 71.43           |   |
| 6   | 23                     | 13 56.52           |   | 13 56.52            |   | 18 69.57           |   |
| 7   | 19                     | 9 47.37            |   | 11 89.47            |   | 12 63.16           |   |
| 8   | 15                     | 7 46.67            |   | 11 73.33            |   | 9 60.00            |   |
| 9   | 19                     | 12 63.16           |   | 10 52.63            |   | 14 73.68           |   |
| 10  | 21                     | 11 52.38           |   | 11 52.38            |   | 12 57.14           |   |
| Total | 208                  | 114 54.81          |   | 127 61.06           |   | 133 63.94          |   |

**Note:** LG, Linkage group; NP: No. of observed polymorphic markers; Pol. (%), Polymorphism percentage.
**Table 4** Segregation pattern of *crtRB1*, *lcyE* and *opaque2*.

| S. No. | Cross                              | Generations | Range of RPG (%) |
|-------|------------------------------------|-------------|-----------------|
| 1     | HKI161 × HP704-23                  | BC1F1       | 71.49–80.26     |
|       |                                    | BC2F1       | 85.53–88.16     |
|       |                                    | BC2F2       | 88.60–92.98     |
| 2     | HKI163 × HP704-22                  | BC1F1       | 70.47–75.98     |
|       |                                    | BC2F1       | 83.07–86.22     |
|       |                                    | BC2F2       | 83.86–91.73     |
| 3     | HKI193-1 × HP704-23                | BC1F1       | 70.68–80.83     |
|       |                                    | BC2F1       | 85.71–90.60     |
|       |                                    | BC2F2       | 87.97–92.11     |
| 4     | HKI193-2 × HP704-22                | BC1F1       | 72.98–80.65     |
|       |                                    | BC2F1       | 85.89–88.31     |
|       |                                    | BC2F2       | 86.69–91.53     |

2014 and winter season 2014–15, respectively. The RPG recovery in the selected plants ranged from 83.86 to 92.98% across four crosses (Table 4, Figure 2). Selection of morphological traits helped in deriving phenotypically similar progenies with their original versions. However, in case of HKI193-2-based progenies, *CC/LL/o2o2* possessed undesirable characteristics of tip opening of ear and irregular grain arrangements, thus were not selected. Instead, progenies of genetic constitution “*CC/LL/L/o2o2*” with desirable characteristics was selfed to develop BC2F3 population in winter season 2014–15 to recover “*CC/LL/o2o2*” genotypes (Table S2). The segregation pattern of *crtRB1* locus showed deviation from the expected Mendelian ratio in all populations across the generations of four crosses, but *lcyE* segregated as per the expectation. *o2* gene was also showed Mendelian monohybrid pattern of inheritance except in cross-II.

**Evaluation of Introgressed Inbreds**

The carotenoid analysis of MABB-derived selected introgressed progenies of HKI161, HKI163, HKI193-1, and HKI193-2 showed a significant increase over their respective recurrent parents (Table 6). The proA concentration among improved inbreds ranged from 7.38 to 13.59 µg/g, compared to 1.65–2.04 µg/g among recurrent parents (Table 6). An average of 6-fold increase in proA was recorded among introgressed progenies. The lysine and tryptophan among the MABB-derived progenies (lysine: 0.274–0.394%, tryptophan: 0.071–0.084%) were at par with their respective parental lines (Table 6). The plant phenotypic characteristics and grain yield attributing traits of the introgressed lines were similar to their respective recurrent parents (Table 7). The opaneness of 25–50% was recorded among introgressed progenies of HKI161 and HKI163, while 50–75% and 95–100% was observed among HKI193-1 and HKI193-2-based introgressed lines, respectively. The degree of opaneness is similar to proportion observed among the recurrent parents.

**Evaluation of Reconstituted Hybrids**

The proA among reconstituted hybrids showed an average of 4.5-folds increase over their original versions. The proA of the newly
TABLE 6 | Biochemical evaluation of selected introgressed progenies with their respective recurrent and donor parents.

| S. No. | Genotype       | proA (%) | FC     | RPG (%) | Lysine (%) | Tryptophan (%) |
|--------|----------------|----------|--------|---------|------------|----------------|
| 1.     | HK161-24-62-53-38 | 13.09    | 6.4    | 91.23   | 0.346      | 0.077          |
| 2.     | HK161-24-62-53-61 | 12.13    | 5.9    | 92.54   | 0.339      | 0.075          |
| 3.     | HK161-24-62-53-67 | 13.59    | 6.6    | 90.35   | 0.322      | 0.081          |
| 4.     | HK161 (FP)       | 2.04     | 0.308  | 0.076   |            |                |
| 5.     | HK163-2-90-10-7  | 7.38     | 4.5    | 91.34   | 0.345      | 0.080          |
| 6.     | HK163-2-90-17-41 | 7.98     | 4.8    | 91.34   | 0.342      | 0.075          |
| 7.     | HK163-2-90-17-60 | 9.32     | 5.6    | 90.16   | 0.314      | 0.084          |
| 8.     | HK163 (FP)       | 1.65     | 0.347  | 0.082   |            |                |
| 9.     | HK193-1-1-8-5-25 | 10.21    | 5.6    | 91.73   | 0.322      | 0.081          |
| 10.    | HK193-1-1-8-5-38 | 11.35    | 6.2    | 91.35   | 0.347      | 0.071          |
| 11.    | HK193-1-1-8-5-116| 10.50    | 5.7    | 92.11   | 0.274      | 0.080          |
| 12.    | HK193-1 (RP)     | 1.84     | 0.323  | 0.078   |            |                |
| 13.    | HK193-2-10-8-34-46-10 | 11.07 | 6.3 | 90.32 | 0.394 | 0.072 |
| 14.    | HK193-2-10-8-34-52-48 | 12.18 | 7.0 | 91.13 | 0.306 | 0.079 |
| 15.    | HK193-2-10-8-34-68-34 | 11.37 | 6.5 | 91.53 | 0.366 | 0.074 |
| 16.    | HK193-2 (RP)     | 1.74     | 0.304  | 0.071   |            |                |
| 17.    | HP704-22 (DP)    | 16.05    | 0.176  | 0.028   |            |                |
| 18.    | HP704-23 (DP)    | 15.28    | 0.192  | 0.035   |            |                |

proA, provitamin A; FC, Fold Change; RP, Recurrent Parent; DP, Donor Parent.

derived hybrids ranged from 9.25 to 12.88 µg/g compared to 2.14–2.48 µg/g among the original hybrids (Figure 3). The mean proA in HQPM1-, HQPM4-, HQPM5-, and HQPM7-based reconstituted hybrids was 9.95, 10.47, 9.63, and 12.27 µg/g, respectively (Figure 3). The proA fold change was as high as 4.6 times in HQPM1-B over its original hybrid, while HQPM4-A, HQPM5-C, and HQPM7-B had 4.7-, 4.7-, and 5.1-fold increase in proA over their respective original hybrids. The lysine and tryptophan among the MABB-derived versions was at par with their respective original versions of hybrid (Figure 4). Among reconstituted hybrids, lysine ranged from 0.291 to 0.365%, while tryptophan varied from 0.072 to 0.085%. The improved hybrids showed high degree of resemblance for agronomic traits with their respective original hybrids across locations (Table 8 and Table S4). The grain yield and attributing traits of MABB-derived hybrids were also at par with their respective original versions (Table 8, Table S4).

DISCUSSION

Normal maize endosperm contains low lysine and tryptophan, however their level is elevated by almost double in QPM genotypes due to recessive o2 present on chromosome 7 (Mertz et al., 1964; Vasal, 2000). However QPM like traditional normal maize genotypes also possesses very low proA carotenoids (Gupta et al., 2015). Animal's metabolism cannot synthesize lysine, tryptophan, and proA in their body, therefore the requirement is to be met from food sources (Pixley et al., 2013). Mutant version of crtRB1 and lcyE enhances proA level and makes maize grain more nutritious for human/ animal consumption (Harjes et al., 2008; Yan et al., 2010; Babu et al., 2013). The present study used MABB for combining the favorable
The difference in segregation distortion (SD) was observed between recurrent and donor parents. Also, improved progenies did not find any SD thus necessitates assaying of large population with an exception in one group. SD thus necessitates assaying of large population with an exception in one group. Similarly, reduced segregation distortion was observed in both recurrent and donor parents.

Morphological characterization of improved lines with their respective recurrent parents.

| S. No. | Genotypes         | MF (days) | FF (days) | PH (cm) | EH (cm) | EL (cm) | EW (cm) | NR  (no.) | NKR (no.) | TW  (g) |
|--------|-------------------|-----------|-----------|---------|---------|---------|---------|-----------|-----------|--------|
| 1.     | HKI161-24-62-53-38| 53.00     | 56.00     | 181.00  | 72.33   | 11.47   | 3.10    | 12.67     | 19.00     | 31.17  |
| 2.     | HKI161-24-62-53-61| 54.00     | 56.00     | 185.33  | 67.33   | 12.90   | 3.07    | 12.00     | 20.00     | 31.27  |
| 3.     | HKI161-24-62-53-67| 53.00     | 56.00     | 188.33  | 72.33   | 12.87   | 2.93    | 10.67     | 17.33     | 29.80  |
| 4.     | HKI161 (RP)       | 53.00     | 56.00     | 181.67  | 69.33   | 12.07   | 3.13    | 12.00     | 20.67     | 31.57  |
| 5.     | HKI63-2-90-10-7   | 60.00     | 65.00     | 170.76  | 81.00   | 13.17   | 3.10    | 12.67     | 24.33     | 27.03  |
| 6.     | HKI63-2-90-17-41  | 60.00     | 64.00     | 172.00  | 77.67   | 11.80   | 2.67    | 12.67     | 23.33     | 25.37  |
| 7.     | HKI63-2-90-17-60  | 61.00     | 65.00     | 167.00  | 76.67   | 11.53   | 2.33    | 11.33     | 20.00     | 27.27  |
| 8.     | HKI63 (RP)        | 60.00     | 64.00     | 173.33  | 76.00   | 11.57   | 2.73    | 12.00     | 22.33     | 27.37  |
| 9.     | HKI93-1-1-8-5-25  | 58.00     | 62.00     | 168.33  | 58.33   | 9.77    | 2.10    | 10.67     | 19.63     | 20.47  |
| 10.    | HKI93-1-1-8-5-38  | 59.00     | 63.00     | 161.00  | 49.33   | 10.33   | 2.03    | 11.33     | 19.67     | 18.97  |
| 11.    | HKI93-1-1-8-5-116 | 58.00     | 62.00     | 159.00  | 52.67   | 11.67   | 2.40    | 12.00     | 22.00     | 19.63  |
| 12.    | HKI93-1 (RP)      | 59.00     | 63.00     | 161.00  | 50.00   | 10.13   | 2.27    | 11.33     | 18.67     | 19.47  |
| 13.    | HKI93-2-10-8-34-46-10 | 56.00 | 60.00     | 163.33  | 71.33   | 8.83    | 2.23    | 10.67     | 17.33     | 19.60  |
| 14.    | HKI93-2-10-8-34-52-46 | 56.00 | 60.00     | 161.33  | 74.67   | 9.67    | 2.40    | 12.33     | 22.67     | 19.40  |
| 15.    | HKI93-2-10-8-34-68-34 | 58.00 | 62.00     | 168.67  | 76.67   | 10.93   | 2.43    | 11.33     | 20.67     | 16.63  |
| 16.    | HKI93-2 (RP)      | 56.00     | 59.00     | 166.67  | 70.00   | 10.17   | 2.43    | 12.67     | 20.33     | 18.23  |

MF: days to 50% male flowering; FF: days to 50% female flowering; PH: plant height; EH: ear height; EL: ear length; EW: ear width; NR: number of rows; NKR: number of kernels per row; TW: 100-seed weight; SE: Standard Error.

Introgressed inbreds possessed 5–7-folds more proA than their respective recipient parents, while the reconstituted hybrids had 4–5-folds higher proA over their original versions. Expression analysis revealed that mutant crtRB1-transcripts was drastically reduced, leading to lesser amount of β-carotene and lesser conversion of β-carotene to β-carotene biosynthesis pathway (Harjes et al., 2008). The difference in expression levels of crtRB1 and lcyE genes was significant in endosperm, but not in embryos and leaves (Babu et al., 2013). The cumulative advantage of a favorable allele of both the genes (crtRB1 and lcyE) occur in low frequency in the maize germplasm (Azmach et al., 2013; Babu et al., 2013; Muthusamy et al., 2015; Gebremeskel et al., 2017). Even in association mapping panel used by Harjes et al. (2008) and Yan et al. (2010) did not find any genotypes with favorable allele of both the genes (crtRB1 and lcyE).

The range of proA concentration was observed among both MABB-derived inbreds and hybrids, despite having the same allele of crtRB1 and lcyE. This variation is possibly due to varied interaction of crtRB1 and lcyE with the genome (Babu et al., 2013; Muthusamy et al., 2014). Also, improved progenies of four crosses revealed kernel proA concentration lower than their respective donor parents (Table 2). This suggests that other allelic combinations of the genes may play a role in the variation in proA content.
genetic loci or QTLs apart from favorable alleles of the *crtRB1* and *lcyE* genes, contribute to the accumulation of proA (Wong et al., 2004; Chander et al., 2008; Zhou et al., 2012; Kandianis et al., 2013). Current study has achieved 70% of target level 15 µg/g proA in reconstituted hybrids (mean: 10.58 µg/g) which emphasize the need for further introgression of genetic loci like *crtRB3*, *CCD1*, and *ZEP1* (Zhou et al., 2012; Suwarno et al., 2015).

The nutritional benefit of QPM with enhanced lysine and tryptophan were also conserved in the MABB-derived lines and their reconstituted hybrids. The *o2* leads to reduction of zein proteins, with a concurrent increase in non-zein proteins rich in lysine and tryptophan (Ueda et al., 1992). *o2* also down regulates the synthesis of *lysine ketoglutarate reductase* resulting in increased levels of free lysine (Kemper et al., 1999). Besides, it is also involved in regulation of various lysine-rich proteins and enzymes (Jia et al., 2013). The variation of lysine and tryptophan observed in the *o2*-based introgressed progenies is due to various modifier loci including *opaque6* that affect regulation of amino acid biosynthesis (Wu et al., 2002; Yang et al., 2005; Pandey et al., 2015; Sarika et al., 2017). Similarly, the variation for lysine and tryptophan among *o2*-introgressed progenies was also observed by Gupta et al. (2013) and Hossain et al. (2017) in their MABB programmes.

The grain yield of reconstituted hybrids was also at par with the original hybrids. The similarity was due to indirect selection of loci for yield potential and various agronomic traits through background selection. Yield has not been used as the criterion of selecting the segregants, however the introgressed progenies led to the development of heterotic hybrids which were similar to the original hybrids (Gupta et al., 2013; Muthusamy et al., 2014; Hossain et al., 2017). The study thus implemented a successful demonstration of MABB augmented with stringent phenotypic selection for agro-morphological characters. The present investigation was the first report of combining favorable alleles of *crtRB1*, *lcyE*, and *o2* in a single genetic background. During the year 2017, *o2*, and *crtRB1*-based “Pusa Vivek QPM9 Improved” maize hybrid with high proA, lysine and tryptophan has been released by ICAR in India (Muthusamy et al., 2014). Pusa Vivek QPM9 Improved provides an average grain yield of 5,588 and 5,916 kg/ha in Zone-I and Zone-IV, respectively (Annual Progress Report Kharif Maize, 2016). In comparison, the newly developed proA rich QPM hybrids in the present study possessed higher average grain yield (mean: 7,314 kg/ha). Moreover, these hybrids (Zone-II, III, IV, and V, Table 1) are also adapted to diverse agro-ecological zones.

The improved inbreds thus developed here can be used as donor lines for simultaneous introgression of *o2*, *crtRB1*, and *lcyE* in the breeding programme. Further, the improved
inbreeds can be crossed among them to generate different F₂ populations, where from new inbreeds with high proA, lysine and tryptophan can be derived using pedigree method. The nutritionally improved hybrids can be grown for cultivation for commercial usage of biofortified grains as food and feed. The significance of biofortified maize for human health has very well observed in many countries (Bouis and Saltzman, 2017). The benefit of QPM in human health and poultry birds is also well documented (Gunaratna et al., 2010; Panda et al., 2014). Biofortified orange maize was found to be as efficacious as a vitamin A supplement in children (Gannon et al., 2014). Dubey et al. (2017) using caco-2 cell model demonstrated that proA rich maize hybrids having ctrlRI allele possessed enhanced bioavailability of β-carotene. Chickens fed with biofortified maize produced eggs rich in proA (Liu et al., 2012; Heying et al., 2014; Moreno et al., 2016; Sowa et al., 2017). A study further revealed that proA biofortified fed chickens had higher redness and yellowness and lower lightness in the meat and skin color than white maize fed chickens (Odunitan-Wayas et al., 2016). Thus, both direct consumption through foods and indirect consumption through chicken - eggs and -meats, proA rich maize contributes to nutritional security. Lvidini and Fiedler (2015) demonstrated the great promise of proA rich maize for becoming a highly cost-effective strategy for reducing malnutrition. Biofortified high yielding maize hybrid rich in proA, lysine and tryptophan nutrients would be a sustainable delivery tools to overcome micronutrient malnutrition.

CONCLUSIONS

We report here the development of four maize hybrids using marker-assisted stacking of o₂, ctrlRI, and lcyE. The hybrids were evaluated at two locations and provided similar grain yield potential of the original hybrids. The inbreds with elevated lysine, tryptophan and proA concentration can be used as potential donors for development of nutrient rich maize cultivars in future breeding programmes. The biofortified maize hybrids enriched with proA, lysine and tryptophan possess great potential to simultaneously alleviate vitamin A deficiency and protein-energy malnutrition across the world.

AUTHOR CONTRIBUTIONS

Conduct of all experiments: RZ; Development of segregating progenies: FH and VM; Morphological characterization: FH and JB; Phenotyping for kernel quality: VM, AB and SS; Analysis on kernel modification: HC; Statistical analyses: FH and NT; Phenotyping for kernel quality: VM, AB and SS; Analysis on kernel modification: HC; Statistical analyses: FH and NT; Drafting of the manuscript: RZ and FH; Designing of the experiment: FH and HG.

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### TABLE 8 | Combined analysis of reconstituted hybrids along with original hybrids across the locations.

| S. No. | Genotypes  | MF (days) | FF (days) | PH (cm) | EH (cm) | EL (cm) | EW (cm) | NR (no.) | NKR (no.) | TW (g) | GY (kg/ha) |
|--------|------------|-----------|-----------|---------|---------|---------|---------|----------|-----------|--------|------------|
| 1      | HQPM1      | 59.00     | 60.50     | 180.75  | 81.90   | 15.10   | 3.65    | 14.30    | 28.00     | 25.25  | 6559.00    |
| 2      | HQPM1-A    | 57.00     | 59.75     | 180.30  | 79.40   | 15.60   | 3.80    | 14.00    | 30.30     | 26.90  | 6770.00    |
| 3      | HQPM1-B    | 57.50     | 59.50     | 185.95  | 80.65   | 15.50   | 3.60    | 14.50    | 28.20     | 27.40  | 6843.00    |
| 4      | HQPM1-C    | 58.50     | 60.50     | 174.70  | 76.55   | 15.30   | 3.90    | 14.40    | 30.00     | 26.40  | 6917.50    |
| 5      | HQPM4      | 60.00     | 60.75     | 197.20  | 86.25   | 17.10   | 4.05    | 14.05    | 33.35     | 30.20  | 7315.00    |
| 6      | HQPM4-A    | 58.75     | 58.75     | 195.00  | 82.50   | 16.90   | 4.00    | 14.30    | 32.05     | 29.30  | 7354.00    |
| 7      | HQPM4-B    | 57.50     | 59.25     | 199.40  | 86.25   | 17.45   | 3.95    | 14.50    | 32.75     | 29.20  | 7239.00    |
| 8      | HQPM4-C    | 58.00     | 57.75     | 189.60  | 81.75   | 16.90   | 4.00    | 14.15    | 31.50     | 30.20  | 7468.50    |
| 9      | HQPM5      | 58.25     | 59.50     | 180.95  | 76.25   | 15.40   | 3.70    | 14.70    | 28.15     | 27.85  | 7793.50    |
| 10     | HQPM5-A    | 56.50     | 57.25     | 197.20  | 83.80   | 15.75   | 3.95    | 14.85    | 30.90     | 28.90  | 7605.00    |
| 11     | HQPM5-B    | 58.25     | 59.00     | 186.25  | 84.70   | 15.65   | 4.00    | 14.00    | 30.90     | 27.10  | 7388.00    |
| 12     | HQPM5-C    | 58.50     | 59.50     | 191.05  | 84.05   | 15.50   | 4.15    | 15.35    | 29.25     | 26.65  | 7728.00    |
| 13     | HQPM7      | 59.25     | 59.75     | 187.60  | 76.60   | 16.20   | 3.95    | 14.35    | 29.35     | 29.80  | 7513.50    |
| 14     | HQPM7-A    | 58.50     | 59.50     | 188.75  | 80.00   | 16.40   | 4.00    | 14.35    | 31.25     | 29.30  | 7632.00    |
| 15     | HQPM7-B    | 59.25     | 60.25     | 194.05  | 85.30   | 16.70   | 4.05    | 14.50    | 30.80     | 30.45  | 7415.50    |
| 16     | HQPM7-C    | 57.25     | 58.50     | 183.90  | 80.20   | 16.75   | 3.90    | 14.60    | 31.10     | 27.65  | 7352.50    |

SE 0.96 1.32 5.31 4.24 0.50 0.16 0.49 0.89 0.78 644.67

MF, days to 50% male flowering; FF, days to 50% female flowering; PH, plant height; EH, ear height; EL, ear length; EW, ear width; NR, number of rows; NKR, number of kernels per row; TW, 100-seed weight; GY, grain yield; DL, Deh; DW, Dharaw; HQPM1-A, HKI193-1-1-8-5-116 × HKI163-2-90-17-60; HQPM1-B, HKI193-1-1-8-5-116 × HKI163-2-90-17-11; HQPM4-A, HKI193-2-10-8-34-66-34 × HKI161-24-62-53-38; HQPM4-B, HKI193-2-10-8-34-52-46 × HKI161-24-62-53-38; HQPM5-A, HKI193-1-1-8-5-38 × HKI163-2-90-17-41; HQPM5-B, HKI193-2-10-8-34-66-34 × HKI161-24-62-53-38; HQPM5-B, HKI163-2-90-10-7 × HKI161-24-62-65-67; HQPM5-B, HKI163-2-90-17-41 × HKI161-24-62-53-67; HQPM7-A, HKI193-1-1-8-5-116 × HKI161-24-62-53-61; HQPM7-B, HKI193-1-1-8-5-25 × HKI161-24-62-53-38; HQPM7-C, HKI193-1-1-8-5-38 × HKI161-24-62-53-61; SE, Standard Error.
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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00178/full#supplementary-material

**Figure S1** | Foreground selection for crtbR1, lcyE, and o2 in BcF2 generation. Star indicates plants heterozygous for crtbR1/lcyE and homozygous for o2 allele. RP, Recurrent Parent; DP, Donor Parent.

**Table S1** | Details of genetic materials used in MABB.

**Table S2** | Details of populations generated under MABB.

**Table S3** | List of SSR markers used in background selection.

**Table S4** | Morphological characterization of reconstituted hybrids along with their original versions.

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Conflict of Interest Statement: 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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