Enrichment of Kernel β-carotene in Maize Hybrids using Marker-assisted Backcross Breeding Strategy

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Introduction

Micronutrient malnutrition, predominantly caused by inadequate consumption of minerals and vitamins, is one of the major deficiency-related health problems in the developing world (Kennedy et al. 2003).Vitamin A deficiency (VAD) affects over 250 million people worldwide and is the primary cause for night blindness among children (WHO 2009). VAD in young children, pregnant women and lactating mothers, the most vulnerable groups, results in reduced resistance to infectious diseases accounting for about 70 percent of the childhood deaths globally (Black et al. 2008). Strategies like supplementation, dietary diversification and fortification of foods have been proposed to overcome micronutrient malnutrition related health problems (West 2000). However, none of these approaches had been viable in the long run, due to an ineffective distribution system, poor infrastructure and/or these solutions are not affordable for resource-poor people (Tanumihardjo et al. 2007). The biofortification approach is cost-effective and sustainable as micronutrients can be delivered in foods consumed by these groups. Therefore, biofortified food crops could serve as an effective way to provide essential micronutrients to a consumer whose diet is heavily based on the staple grains (Ortiz-Monasterio et al. 2007).

The yellow kernel maize exhibits tremendous natural variation for kernel carotenoids that may be exploited through plant breeding (Buckner et al. 1990). Maize is one of the three-most important staple food crops in the world, serving more than one billion people in Africa, Meso-America and many Asian countries (Gupta et al. 2009; Shiferaw et al. 2011). Maize is also an important cereal in Asia, where more than half of the produce is used by the livestock industry, resulting in rapid economic growth (Gupta et al. 2013a). Maize has been targeted for biofortification for other nutrients and these efforts were largely successful (Prasanna et al. 2001; Vasal 2001; Babu et al. 2005; Atlin et al. 2011; Gupta et al. 2013a; Gupta et al. 2013b). Yellow maize with diverse end- uses and wide- variability for carotenoids, holds promise for provitamin A biofortification to alleviate widespread VAD in humans.

The biggest challenge to breed provitamin A enriched maize is large scale phenotyping of kernel carotenoids in maize kernels. Quantifying the provitamin A carotenoids using HPLC is time-consuming and expensive. Therefore, breeding programs will benefit greatly from marker-assisted selection (MAS) by reducing the need for phenotypic assays. By selecting for the genes causing β-carotene enhancement in the carotenoid biosynthesis pathway using molecular markers, concentration of β-carotene can be increased in the maize endosperm. MAS has been advocated as a highly efficient breeding method as it makes rapid- and precise- selection of the individuals possessing the targeted gene (Ribaut and Hoisington 1998). It is found to be the most effective way of transferring gene(s) to an otherwise, agronomically superior, variety or parental line (Singh et al. 2012).

Among the genes involved in the carotenoid biosynthesis pathway, *phytoene synthase1* (*PSY1 or Y1*), plays a pivotal role by condensing two geranyl-geranyl pyrophosphate molecules into one molecule of phytoene (Buckner et al. 1990). Plants with *Y1* gene produce carotenoids and determine the variation for kernel color in maize (Buckner et al. 1990). The first branch point of the pathway occurs at cyclization of lycopene, where the *lycopene epsilon cyclase* (*lcyE*) gene in association with other genes convert more lycopene to β, ε branch that produces more α-carotene and lutein. Naturally existing mutant alleles of *lcyE* with reduced expression has been identified that diverts more lycopene to β, β branch, thereby producing more β-carotene, β-cryptoxanthin and zeaxanthin (Harjes et al. 2008).

Though the favorable *lcyE* allele increases the proportion of β-carotene in the pathway, a large amount of that gets hydroxylated by *β-carotene hydroxylase* (*crtRB1*) gene to produce β-cryptoxanthin (50 percent provitamin A activity) and zeaxanthin (no provitamin A activity). Therefore, for enhanced accumulation of provitamin A, blocking of β-carotene

hydroxylation can effectively increase the levels of β-carotene relative to β-cryptoxanthin and the downstream zeaxanthin. Natural genetic variation in *crtRB1* gene has been reported (Yan et al. 2010), and large-scale validation experiments carried out suggested that *crtRB1* 3'TE favorable allele alone cause two to ten-fold variation in the β-carotene concentration in maize (Babu et al. 2013). PCR-based co-dominant markers were identified for *crtRB1* 3'TE polymorphisms which would help in rapid improvement of provitamin A through MAS (Yan et al. 2010; Babu et al. 2013; Vignesh et al. 2013).

Considering the importance of provitamin A biofortification and potential of *crtRB1* gene in enhancing β-carotene concentration of maize, the present investigation was undertaken to study the genetic variability for kernel β-carotene among diverse maize inbreds; and to introgress the favorable *crtRB1* 3'TE allele into elite inbred parents of agronomically-superior commercial maize hybrids, through marker-assisted backcross breeding (MABB).

Materials and methods

Genetic materials

To estimate the genetic variability, a diverse set of 95 inbreds, from Indian- and CIMMYT- maize breeding programs, with wide variation for kernel color, were selected and evaluated in a replicated trial. A new set of inbred lines (HP-) developed under the CIMMYT-Maize HarvestPlus program, having *crtRB1* favorable allele, were also evaluated in the study. Self-pollinated ears were used to estimate kernel β-carotene. Inbreds were visually scored for their kernel color and were correlated with the kernel β-carotene.

For marker-assisted introgression of *crtRB1* 3'TE allele, seven elite inbreds (VQL1, VQL2, V335, V345, HKI1105, HKI323 and HKI161) that are parents of four-commercial maize hybrids (Vivek QPM 9, Vivek Hybrid 27, HM 4 and HM 8) were selected (Table 1). The hybrids were selected based on their maturity group, grain-yield potential and wider adaptation. Among the seven recurrent parents, VQL1, VQL2 and HKI161 are quality protein maize (QPM) genotypes having *opaque2* allele with high-lysine and tryptophan in the maize endosperm. The inbreds developed under the CIMMYT-HarvestPlus program, with *crtRB1* 3'TE favorable allele and high-kernel β-carotene, were used as donors for introgression of the target gene.

Table 1. Details of hybrids targeted for β-carotene enrichment

S. No.	Hybrid	Parentage	Maturity
1	Vivek QPM 9	VQL1 × VQL2	Extra early
2	Vivek Hybrid 27	V335 × V345	Extra early
3	HM 4	HKI1105 × HKI323	Medium
4	HM 8	HKI1105 × HKI161	Medium

Breeding scheme for marker-assisted introgression of target gene

The seven elite inbreds were crossed as female parent with their donor inbreds (Table 2). A two- generation-based, marker-assisted, backcross-breeding strategy, was employed for the introgression of the *crtRB1* favorable allele. Heterozygotes for the *crtRB1* alleles were selected in BC_1F_1 and BC_2F_1 generations, while homozygotes were selected in BC_2F_2. BC_2F_3 progenies were crossed to reconstitute the original hybrids. The experimental hybrids, along with their original versions, were then tested at two diverse locations (viz., IARI, New Delhi in Northern India and IARI-Regional Research Centre, Dharwad in Southern India) to test their agronomic performance during rainy season 2013. Self-pollinated F_2 seeds, harvested from each of the hybrid combinations, were used for estimation of concentration of kernel β-carotene. Seeds of the introgressed inbreds were also evaluated for estimation of kernel β-carotene.

Table 2. Mean concentration of kernel β-carotene in the introgressed progenies and their recurrent and donor parents

S. No.	Recurrent inbred	β-carotene (µg/g)	Donor inbred	β-carotene (µg/g)	Introgressed Provitamin A version	β-carotene (µg/g)
1	VQL1	1.4	HP465-43	17.8	VQL1-PV	17.0
2	VQL2	1.3	HP465-41	16.8	VQL2-PV	16.3
3	V335	1.3	HP465-30	16.5	V335-PV	16.4
4	V345	1.5	HP465-35	13.9	V345-PV	11.0
5	HKI1105	1.3	HP467-6	14.6	HKI1105-PV	13.7
6	HKI323	1.5	HP467-4	11.3	HKI323-PV	09.7
7	HKI161	1.3	HP467-13	16.9	HKI161-PV	15.6

Molecular marker assay

Polymerase chain reaction (PCR) was performed using *crtRB1* 3'TE gene-specific markers in each of the backcross- and selfed- generations, for selection of the targeted gene. Polymerase chain reaction (PCR) amplification was carried out using the standard cycle conditions, as given by Yan et al. (2010). Amplified fragments were resolved using agarose gel electrophoresis and were scored for the presence of favorable *allele 1* (543 bp) (Yan et al. 2010). Since three of the seven recurrent parents (VQL1, VQL2 and HKI161) are QPM genotypes, foreground selection was also carried out for a simple sequence repeat (SSR) marker (*umc1066*) linked to *opaque2* gene (Gupta et al. 2013a). Further, a set of ~200 SSR markers covering all the 10 chromosomes of maize, were used for a polymorphism survey between the recurrent and the donor parents. The polymorphic primers were employed in each of the backcross generations, to recover the recurrent parent genome (RPG).

Biochemical analysis for estimation of β-carotene

Self-fertilized cobs from each genotype were harvested individually with the husk and the grains separated and stored in a dark setting at 4°C, until the carotenoid extraction, to avoid loss of carotenoids. Carotenoid compounds are sensitive to light, heat and oxygen (Quackenbush 1963) and therefore, sample preparation was done under dark conditions as per the procedure described by Kurilich and Juvick (1999), with minor modifications (Vignesh 2012) and stored in a freezer. Quantification of the β-carotene was done with a Water Alliance HPLC System. Absorbance was measured at 450 nano-meter (nm) for detection of β-carotene.

Results and discussion

Genetic variability for kernel β-carotene

Multi-location evaluation of the set of 95 maize inbred lines revealed low-level of variation (0.02-1.75 µg/g) for kernel β-carotene (mean: 0.45 µg/g) across locations. The proportion of β-carotene, compared to all of the carotenoids, was ~3 percent, when compared to other carotenoids (Figure 1). Chander et al. (2008) also observed a similar trend of carotenoids with less provitamin A while evaluating a set of Chinese germplasm. β-carotene is an intermediate in the pathway, leading to the greater synthesis of downstream non-provitamin A (lutein and zeaxanthin) carotenoids (Yan et al. 2010). In contrast, the average concentration of β-carotene in the CIMMYT-HarvestPlus inbreds with *crtRB13'TE* favorable allele, was 12.12 µg/g, and comprised 81 percent of the total carotenoids (Figure 1). Inbreds having ~15 µg/g of provitamin A, the target level set by HarvestPlus to meet the recommended dietary allowance, were identified in the study. These inbreds possess favorable allele of *crtRB1* gene that increases the concentration of β-carotene by preventing its further hydroxylation to β-cryptoxanthin and zeaxanthin in the pathway (Yan et al. 2010). β-carotene, the major provitamin A carotenoid, did not show correlation with variation in kernel color, revealing that kernel color is not a reliable indicator to select for high- β-carotene (Harjes et al. 2008). Carotenoid profiling using HPLC is laborious, expensive and time consuming and thus, MAS for *crtRB1*gene is a more effective approach to enrich kernel β-carotene in maize (Yan et al. 2010; Vignesh et al. 2012; Babu et al. 2013) to alleviate VAD worldwide.

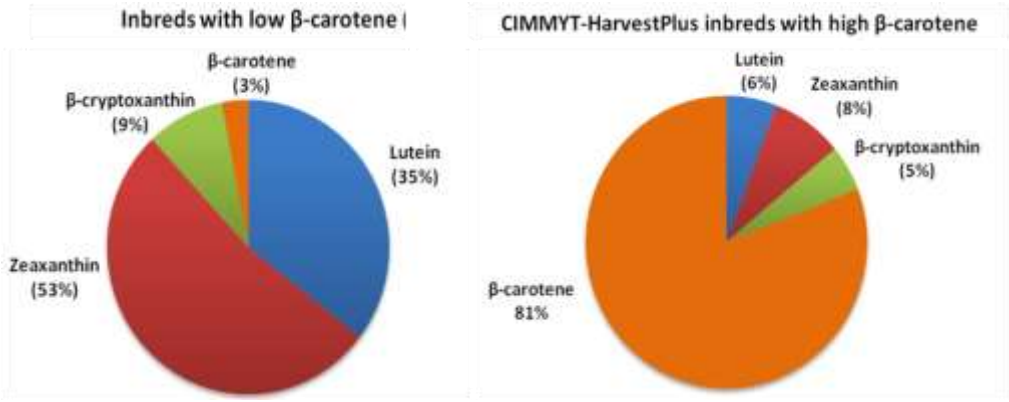

Figure 1. Proportion of different carotenoids observed among low and high- β-carotene inbreds

Parental polymorphism for target gene and genome-based SSR markers

Distinct polymorphism was observed between the recurrent and donor parents for the *crtRB1* 3'TE gene-specific marker. The favorable allele (543 bp) of *crtRB1* 3'TE gene has been reported to have a large and significant effect on enhancing the β-carotene concentration (Yan et al. 2010). Among the seven inbreds targeted in the study, three inbreds (viz. VQL1, VQL2 and HKI161) were QPM inbreds. They showed polymorphism with their respective donor parents for the *opaque2* gene-specific SSR marker *umc1066*. Both the *crtRB1* 3'TE gene-specific marker and the SSR marker *umc1066* are located within the target gene, therefore, individual plants in the populations could be selected directly, without the probability of the occurrence of false positives (Babu et al. 2005; Yan et al. 2010). Of the 200 SSR markers screened between the parents for polymorphism survey, the number of polymorphic markers ranged from 66 in HKI161 × HP467-13 to 82 for V345 × HP465-35. A representative gel showing parental polymorphism survey using SSR markers is shown in figure 2. These polymorphic markers were used for background selection for recovery of the RPG.

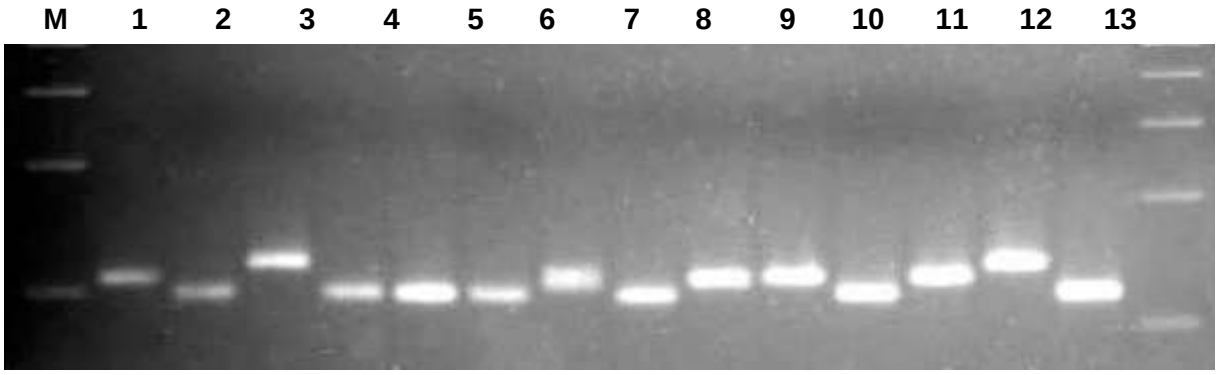

Figure 2. Polymorphism survey between recurrent and donor parents with SSR markers;

1- VQL1; 2- HP465-43; 3- VQL2; 4- HP465-41; 5- V335; 6- HP465-30; 7- V345; 8- HP465-35; 9- HKI1105; 10- HP467-6; 11- HKI323; 12- HP467-4; 13- HKI161; 14- HP467-13; M- DNA ladder

Marker-assisted foreground and background selection

Foreground selection, using the gene-based markers for *crtRB1* favorable allele, identified heterozygous plants in the backcross generations and homozygous plants in the selfed generations (Figure 3). The segregation pattern of *allele 1* showed segregation distortion (SD) in some of the backcross- and selfed-generations spanning seven crosses. Earlier studies have also reported the frequent occurrence of SD for the *crtRB1* allele (Babu et al. 2013). The genetic background of the target allele and the presence of many segregation distortion regions throughout the maize genome (Lu et al. 2002; Babu et al. 2013) could be the reason behind the occurrence of SD and it therefore, necessitates assaying a large number of segregating individuals in order to obtain sufficient numbers of foreground-positive genotypes. Background selection in the BC_2F_1 heterozygous plants using polymorphic SSRs led to the recovery of 86.4 percent RPG in V345 × HP465-35 and recovery of 93.7 percent RPG in VQL1 × HP465-43. Selfing of the selected heterozygotes led to the BC_2F_2 generation.

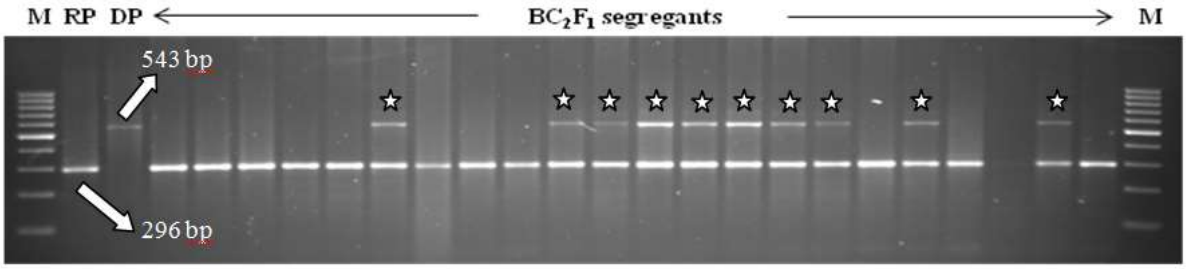

Figure 3. Foreground selection for *crtRB1* 3'TE favorable allele in BC_2F_1 generation using *crtRB1* gene-specific marker; RP- Recurrent parent; DP- Donor parent; 1 to 22- BC_2F_1 segregants; M-100 bp ladder;
☆ indicates positive plants.

Concentration of kernel β-carotene in selected introgressed inbreds and hybrids

Mean kernel β-carotene concentration, among the introgressed inbreds, varied from 9.7 µg/g (HKI323-based progenies) to 17.0 µg/g (VQL1-based progenies) (Table 2). Introgression of *allele 1* led to a maximum (12.6-fold) increase in kernel β-carotene in progeny of V335 × HP465-30. The mean kernel β-carotene, for all the recurrent parents, was 1.4 µg/g, whereas the same was 14.2 µg/g for the introgressed inbreds. Considerable increase in kernel β-carotene, among the introgressed inbreds, suggests that introgression of *allele 1* of the *crtRB1* gene alone has a major effect on accumulation of β-carotene in higher concentrations. A similar trend was reported by Babu et al. (2013) while validating the effect of a favorable allele of the *crtRB1* gene in tropical maize. Substantial increase in the concentration of kernel β-carotene was also observed among the reconstituted hybrids over their respective original hybrids. Mean kernel β-carotene concentration, in the reconstituted hybrids, ranged from 11.50 µg/g (HM 4-based hybrids) to 20.55 µg/g (HM 8-based hybrids) (Table 3).

Table 3. Mean kernel β-carotene concentration and grain-yield of the improved- and original- hybrids

Hybrids	Type	β-carotene (µg/g)	Grain yield (t/ha)*
Vivek QPM 9	Original	2.10	5.60
Vivek QPM 9-PV	Improved	20.13	5.81
Vivek Hybrid 27	Original	2.00	7.40
Vivek Hybrid 27-PV	Improved	16.40	7.32
HM 4	Original	1.90	6.65
HM 4-PV	Improved	11.50	6.93
HM 8	Original	2.60	7.45
HM 8-PV	Improved	20.55	7.43

*based on mean performance at two locations viz. Delhi and Dharwad

The improved versions of other two hybrids (viz. Vivek QPM 9 and Vivek Hybrid 27) recorded a mean kernel β-carotene of 20.13 µg/g and 16.40 µg/g respectively (Table 3). The increase in kernel β-carotene is due to the reduction in the transcript expression of the *crtRB1* gene, which decreases the hydroxylation of β-carotene to further carotenoids in the pathway (Yan et al. 2010; Babu et al. 2013). Significant differences in the accumulation of β-carotene among the introgressed progenies were also observed, which could due to the differential interaction of the introgressed genome with the genes involved in the carotenoid biosynthesis pathway and the genetic background of the recurrent parent (Koide et al. 2011; Singh et al. 2012). Introgressed progenies of all seven crosses showed kernel β-carotene concentration lower than that of their respective donor parents (Table 2), suggesting that the modifier genetic loci with minor effects, contribute to the increase of kernel β-carotene concentration in the donor parent. Many such loci for accumulation of β-carotene and other carotenoids have been reported earlier in maize (Chander et al. 2008b; Wong et al. 2004).

The proportion of tryptophan, in endosperm flour, was estimated in the β-carotene enriched versions of QPM hybrid, Vivek QPM 9 and the average tryptophan concentration in the improved versions of Vivek QPM 9 and the original Vivek QPM 9 was 0.08 percent and 0.09 percent, respectively. The results showed that tryptophan concentrations, in the newly developed β-carotene-rich hybrid versions, was comparable with the original hybrid, Vivek QPM 9. This newly-developed hybrid, thus, provides high- β-carotene, along with high- tryptophan.

Agronomic performance of the reconstituted hybrids

The data on grain-yield and yield-attributing characteristics of the reconstituted hybrids (generated by crossing the improved versions of their parental lines) showed that grain yield was on par with the respective original hybrids. Vivek QPM 9 produced a mean- grain-yield of 5.6 tons/hectare (t/ha^{-1}), whereas the mean- grain-yield of the improved versions of Vivek QPM 9 was 5.8 t/ha^{-1} across two locations (Table 3). Improved hybrids also exhibited similarity for important morphological characteristics of the original hybrids (Choudhary 2014). The newly-developed β-carotene-enriched hybrids possessed grain-yield and agronomic performance on par with their respective original hybrids. Retention of similar grain-yield potential in the improved hybrids, is due to high- recovery of RPG in the parental lines, achieved through background selection. The minor differences in relation to grain-yield and associated traits observed, could be possibly due to interactions of a smaller fraction of the donor genome with the recipient genome (Singh et al. 2012).

Conclusion

VAD is widely prevalent worldwide and developing staple crops like maize with enhanced β-carotene concentration will offer possibilities to reduce the population affected by VAD. The investigation successfully demonstrated that the conversion of elite normal and QPM inbreds into β-carotene rich versions through marker-assisted backcross breeding is possible. HarvestPlus, a CGIAR initiative, has set a target of 15 µg/g of β-carotene in maize kernels to help in alleviating VAD in humans (www.harvestplus.org). The improved hybrids developed through this research, contains significantly greater β-carotene (>15 µg/g) which can be directly utilised in alleviating VAD worldwide. Additionally, these introgressed inbreds may also be used as donors for β-carotene enrichment in biofortification programs.

Acknowledgement

MV and MC are thankful to the Indian Council of Agricultural Research (ICAR) for the Senior Research Fellowship and Junior Research Fellowship enabling pursuit of PhD and MSc degrees, respectively. The financial support from the Department of Biotechnology for the project entitled "Development of micro-nutrient maize through molecular breeding," is gratefully acknowledged. The authors thank Torbert Rocheford (Purdue University, US) and Kevin Pixley (CIMMYT, Mexico) for providing β-carotene-enriched lines and Raman Babu (CIMMYT, India) for his help in standardizing the PCR protocol. We also thank maize breeders of the Directorate of Maize Research, New Delhi; VPKAS, Almora and Uchani Centre of the CCSHAU, Karnal for sharing the inbred lines.

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