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Allelic variations for *lycopene-ε-cyclase* and *β-carotene hydroxylase* genes in maize inbreds and their utilization in β-carotene enrichment programme

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Abstract: Vitamin A deficiency is a global health problem and can be effectively alleviated through crop biofortification. Quantification of carotenoids using high-performance liquid chromatography is expensive and time-consuming, thereby posing a challenge in the selection of genotypes with high provitamin A. Favourable alleles possessing rare genetic variation in *lycopene-ε-cyclase* (*lcyE*) and *β-carotene hydroxylase* (*crtRB1*) genes are associated with higher accumulation of provitamin A, especially β-carotene; and selection of these alleles holds immense promise in reducing large-scale phenotypic assays. Screening of a diverse set of 385 maize inbred lines of indigenous and exotic origin detected the presence of two alleles (amplicon size: 250 and 650 bp) of *lcyE* and three alleles (amplicon size: 296, 543 and 875 bp) of *crtRB1* in the inbred panel. Favourable alleles of both the genes were rare among the traditional maize germplasm; 3.38% of the inbreds possessed the favourable allele (650 bp) of *lcyE*, while 3.90% inbreds had the favourable allele (543 bp) of *crtRB1*. Five inbreds (1.3%) with favourable alleles of both the genes were found. Inbreds with favourable alleles of *crtRB1* and *lcyE* serve as rich genetic resources for effective utilization in the maize biofortification programme.

ABOUT THE AUTHORS

ICAR-Indian Agricultural Research Institute, New Delhi, is a flagship organization for providing food and nutritional security in the country. Our research group pursue research for the alleviation of micronutrient malnutrition by biofortifying maize with micronutrients viz. provitamin A, vitamin E, Fe, Zn and essential amino acids viz. lysine and tryptophan through conventional and molecular breeding approaches. The research report in this paper aimed to identify maize inbreds with favourable alleles of two key genes viz. *lycopene-ε-cyclase* and *β-carotene hydroxylase*, governing higher accumulation of β-carotene, a major provitamin A carotenoid in maize kernel. These inbreds will serve as a rich genetic resource and can be utilized by breeding programmes for enrichment of β-carotene. The outcome of the study would help in developing biofortified maize hybrids to alleviate the widespread vitamin A deficiency in humans.

PUBLIC INTEREST STATEMENT

The work presented in the manuscript describes the promise of maize inbreds with favourable alleles of ***lycopene-ε-cyclase*** and ***β-carotene hydroxylase*** genes for enrichment of kernel β-carotene in maize. This also illustrates its utility for diversification of maize germplasm for kernel carotenoids. The inbreds with the favourable allele and high β-carotene serve as potential genetic resources that can be used for the accelerated development of maize hybrids with high β-carotene using marker-assisted selection. These biofortified maize genotypes will serve as sustainable and cost-effective solutions to alleviate micronutrient malnutrition worldwide.

Subjects: Agriculture and Food; Crop Science; Plant Biotechnology

Keywords: β-carotene; biofortification; *crtRB1*; *lcyE*; maize; provitamin A

1. Introduction

Micronutrient malnutrition or "hidden hunger" has become a major health problem and affects millions of people worldwide. Among them, vitamin A deficiency (VAD) alone affects over 250 million pre-school children and accounts for about 70% of the childhood deaths globally (Vignesh et al., 2012). Young children, pregnant women and lactating mothers are most vulnerable to VAD, as it contributes to maternal death, poor pregnancy and lactation (Black et al., 2008). Besides, it also contributes to the predisposition of several major diseases, such as anaemia, diarrhoea, measles, malaria and respiratory infections (West, 2000). Since vitamin A cannot be synthesized inside the human body, it needs to be provided through diet. Hence, increasing provitamin A carotenoids in staple crops through breeding would be a viable strategy to minimize the adverse effects of VAD (Bouis, Hotz, McClafferty, Meenakshi, & Pfeiffer, 2011).

As compared to other staple cereals, maize possesses tremendous variability for kernel carotenoids (Buckner, Kelson, & Robertson, 1990). Quantifying the provitamin A carotenoid using high-performance liquid chromatography (HPLC) is expensive and time-consuming. Polymerase chain reaction (PCR)-based assay thus would help in identifying genotypes with high β-carotene. Three genes in the carotenoid biosynthesis pathway play a crucial role in the accumulation of various carotenoids in the maize kernel. The first committed step in the pathway is regulated by *phytoene synthase* (*psy*) that converts phytoene to geranylgeranyl pyrophosphate leading to yellow/orange-coloured maize endosperm (Buckner et al., 1990). The first branching point of the pathway is the cyclization of lycopene: *lycopene-ε-cyclase* (*lcyE*) gene, in association with other genes, converts more lycopene to the β, ε branch, which produces more α-carotene and lutein (Harjes et al., 2008). Another key gene in the pathway is *β-carotene hydroxylase* (*crtRB1*) that causes the hydroxylation of α- and β-carotene into the non-provitamin A carotenoids viz. lutein and zeaxanthin, respectively (Vallabhaneni et al., 2009; Yan et al., 2010). Therefore, pathway branching and hydroxylation are key determinants in controlling provitamin A levels of the maize kernel (Harjes et al., 2008; Yan et al., 2010).

Harjes et al. (2008) showed that naturally existing mutant allele at the *lycE* locus alters flux down α-carotene versus β-carotene branches of the carotenoid biosynthesis pathway. Using allele mining strategy, four natural *lcyE* polymorphisms viz. *lcyE* 5′TE (Transposable Element in 5′-untranslated region [UTR]), *lcyE* SNP216 (in exon 1), *lcyE* SNP2238 (in intron 4) and *lcyE* 3′InDel (in 3′-UTR) were identified, of which, the favourable allele of *lcyE* 5′TE causes more increase in provitamin A content (Harjes et al., 2008). Harjes et al. (2008) reported the presence of four alleles in the 5′TE region of the *lcyE* gene viz. *allele 1* (150 + 280 bp), *allele 2* (250 bp), *allele 3* (250 bp + 380 bp) and *allele 4* (650 bp). Of these, *allele 1 and allele 4* are known as favourable alleles for enhancing the β-branch carotenoids by increasing the pathway flux towards β- branch, whereas *allele 2* and *allele 3* cause unfavourable effects. Yan et al. (2010) through association mapping approach detected three polymorphisms viz. 5′TE (in the 5′-UTR), InDel4 (in the coding region) and 3′TE (spanning the sixth exon and 3′-UTR) in *crtRB1* that were significantly associated with the β-carotene concentration and its conversion to β-cryptoxanthin and zeaxanthin in maize kernels (Yan et al., 2010), of which *crtRB1* 3′TE favourable allele alone caused two to tenfold variations in the β-carotene concentration among the inbreds (Babu, Rojas, Gao, Yan, & Pixley, 2013; Muthusamy et al., 2014). The 3′TE polymorphism of *crtRB1* gene that spans the 6th exon and the 3′-UTR that generates three alleles viz. *allele 1* (543 bp; without TE insertion), *allele 2* (296 bp + 875 bp; with 325 bp TE insertion) and *allele 3* (296 bp + 1221 bp + 1880 bp; with 1250 bp TE insertion) were associated with altering β-carotene accumulation (Yan et al., 2010). *Allele 1* is known as a favourable allele for enhancing the β-carotene by reducing transcript expression of the *crtRB1* gene, whereas *allele 2* and *allele 3* cause unfavourable effects. PCR-based co-dominant markers were identified for both *lcyE*- and *crtRB1*-based polymorphisms which can pave way for rapid improvement of provitamin A content in maize kernels through marker-assisted selection (MAS) (Zhang et al., 2012). Thus, the present study was

undertaken to screen a large set of inbreds of indigenous and exotic origin to identify genotypes having favourable alleles of *lcyE* and *crtRB1*, for their utilization in maize provitamin A biofortification programme.

2. Materials and methods

2.1. Plant material

A diverse set of 385 diverse maize inbreds from Indian and CIMMYT research programmes were selected for screening for the presence of favourable alleles of *lcyE* and *crtRB1* (Vignesh, 2012). The panel includes parents of released hybrids, elite inbreds, pre-breeding lines, specialty corns and inbreds newly developed from CIMMYT-HarvestPlus programme. Kernel colour of the selected inbreds varied from pale yellow to dark orange. Inbreds were grown at the ICAR-Indian Agricultural Research Institute (IARI) experimental farm, New Delhi, and were self pollinated. Individual ear in each line was harvested with husk and the grains were stored in darkness at 4°C until carotenoids extraction.

2.2. DNA extraction and PCR assay

DNA was isolated from young seedling of each inbred using standard CTAB procedure (Murray & Thompson, 1980) with minor modifications. PCR was performed using gene-specific markers for both the *lcyE* and *crtRB1* genes. Primers for different genomic regions such as 5′TE of the *lcyE* gene and 3′TE of the *crtRB1* gene were custom synthesized. We used the following set of primers, *lcyE*-5′TE-F: AAGCATCCGACCAAAATAACAG and *lcyE*-5′TE-R: GAGAGGGAGACGAC GAGACAC for *lcyE* 5′TE; *crtRB1*-3′TE-F: ACACCACATGGACAAGTTCG, *crtRB1*-3′TE-R1: ACACTCTGGCCCATGAACAC and *crtRB1*-3′TE-R2: ACAGCAATACAGGGG- ACCAG for the *crtRB1* 3′TE polymorphism. PCR amplification was carried out using the standard cycle conditions as given by Harjes et al. (2008) and Yan et al. (2010). Amplified fragments were separated using agarose gel electrophoresis and were scored for the presence of favourable alleles (Harjes et al., 2008; Yan et al., 2010).

2.3. Carotenoids extraction and quantification of kernel β-carotene

The carotenoids extraction and measurement were carried out as per the procedure described by Kurilich and Juvik (1999) with minor modifications. A 20 µl volume of each sample was manually injected into the Water Alliance HPLC System (Waters Chromatography, Milford, MA) attached with a photodiode array detector (PDA). Six dilutions of β-carotene standard (SIGMA chemicals, USA) were used to make the standard curve and the concentration was detected by standard regression with external standard. The HPLC components include e2695 separation module and the 2996 PDA (Waters Corporation). The system was operated with Empower 2 Software (Waters Corporation). YMC carotenoid C30 column (5 µm, 4.6 × 250 mm; Waters Chromatography, Milford, MA) was used. The mobile phase consisted of methanol: tert-butyl methyl ether (80:20, v v^{-1}) and the flow rate was 1 ml min^{-1}. To maximize detection of carotenoids, absorbance was measured at 450 nm.

3. Results

PCR amplification of the diverse inbreds using gene-specific markers revealed polymorphisms for both *lcyE* (at 5′TE region) and *crtRB1* (at 3′TE region) regulating carotenoid biosynthesis pathway.

3.1. Polymorphism for lycopene epsilon cyclase (lcyE) gene

Among the inbreds screened in the study, 13 inbreds possessed 650 bp amplicon (favourable allele; *allele 4*) within the *lcyE* locus (Table 1; Figure 1). All other 372 inbreds in the panel amplified 250 bp amplicon (unfavourable allele; *allele 2*) in the 5′TE region of the gene. Among the 13 inbreds identified, two inbreds viz. HKI161 and HKI163 were from the Indian maize breeding programme. The other 10 inbreds viz. HP19-33, HP255-4, HP255-8, HP255-20, HP180-25, HP465-19, HP465-27, HP467-3, HP467-15 and HP467-20 belonged to CIMMYT-HarvestPlus programme (Table 1). H16, an inbred (from CIMMYT-Africa) with white endosperm also possessed the favourable allele. The concentration of β-carotene in the yellow inbreds possessing favourable allele ranged from 0.19 to 11.51 µg g^{-1} with a mean of 4.45 µg g^{-1}. The white maize inbred, H16, having recessive *y1* allele (*psy*) did not possess

Table 1. List of inbreds identified with the favourable allele (5′TE polymorphism) of *lcyE* gene (650 bp)

S. No.	Genotype	Pedigree	Source
1	HKI161	CML161 (P25QPM)	CCS-HAU, Uchani
2	HKI163	CML163 (P26QPM)	CCS-HAU, Uchani
3	HP255-4	[CML 312/MAS[MSR/312]-117-2]-B-91-3-B-B/[BETASYN]BC1-2-1-1-1-B-B	CIMMYT-HarvestPlus
4	HP255-8	[[EV7992]C1F2-430-3-3-3-X-7-B-B/CML202]-6-2-2-3-B*3/[BETASYN]BC1-10-1-1-1-1-B-B	CIMMYT-HarvestPlus
5	HP255-20	P72c1 × CML-297 × CL-02410-3-1-1-B-B-B	CIMMYT-HarvestPlus
6	HP180-25	Florida A plus Syn-FS2-2-1-B-B	CIMMYT-HarvestPlus
7	H16	—	CIMMYT-Africa
8	HP19-33	KUI carotenoid syn-FS5-2-B	CIMMYT-HarvestPlus
9	HP465-19	(KUI carotenoid syn-FS11-1-1-B-B-B/(KU1409/DE3/KU1409)S2-18-2-B)-B-2	CIMMYT-HarvestPlus
10	HP465-27	(KUI carotenoid syn-FS17-3-2-B-B-B/(KU1409/DE3/KU1409)S2-18-2-B)-B-1	CIMMYT-HarvestPlus
11	HP467-3	(KUI carotenoid syn-FS11-1-1-B-B-B/(KU1409/DE3/KU1409)S2-18-2-B)-B-2	CIMMYT-HarvestPlus
12	HP467-15	(KUI carotenoid syn-FS11-1-1-B-B-B/(KU1409/DE3/KU1409)S2-18-2-B)-B-3	CIMMYT-HarvestPlus
13	HP467-20	(KUI carotenoid syn-FS17-3-2-B-B-B/(KU1409/DE3/KU1409)S2-18-2-B)-B-1	CIMMYT-HarvestPlus

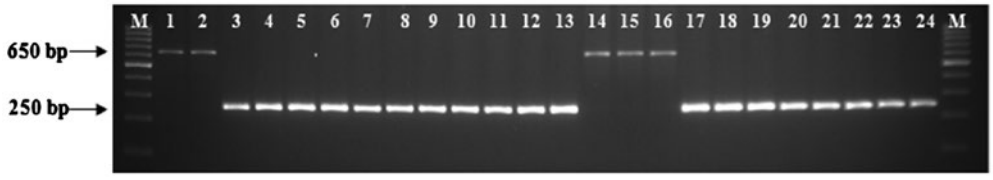

Figure 1. Allelic variations at 5′TE region of *lcyE* gene.

Note: Lane 1, 2, 14, 15 and 16: Inbreds with the favourable allele (*allele 4*: 650 bp amplicon); Lane 3–13, 17–24: Inbreds with the unfavourable allele (*allele 2*: 250 bp amplicon); M: 100 bp DNA ladder.

any β-carotene in the kernel. Among the identified inbreds, only the five new set of HarvestPlus inbreds viz. HP465-19, HP465-27, HP467-3, HP467-15 and HP467-20 had higher concentration of β-carotene (8.61–11.51 µg g^{-1}) and the rest of seven inbreds had less β-carotene (0.19–0.69 µg g^{-1}) despite having the favourable allele.

3.2. Polymorphism for crtRB1 gene

Among the 385 genotypes screened, 15 inbreds amplified 543 bp amplicon (favourable allele; *allele 1*) in the 3′TE region of the *crtRB1* (Table 2; Figure 2). Among the inbreds identified with the favourable allele, SE547, Pant120, Pant125, V372, MGUDM-RIL47, KDMI4, EI116, MGUQPM-MAS4979 and BAJIM 06-10 are from the Indian maize breeding programme. The other inbreds viz. HP465-19, HP465-27, HP467-3, HP467-15 and HP467-20 belong to CIMMYT-HarvestPlus programme (Table 2). Among the other inbreds that amplified the unfavourable alleles, 51 inbreds had *allele 2* and 319 inbreds had *allele 3*. Estimation of β-carotene in the inbreds (having favourable allele) through HPLC revealed that 14 yellow inbreds had a mean β-carotene of 4.49 µg g^{-1} with a range of 0.41–11.51 µg g^{-1}. Of the 15 inbreds identified in the study, surprisingly the inbred EI116 with white endosperm and absolutely no carotenoid also had the favourable allele for *crtRB1* 3′TE gene (Table 2). Among the other 14 inbreds, only five in the new set of HarvestPlus inbreds viz. HP465-19, HP465-27, HP467-3, HP467-15 and HP467-20 had higher concentration of β-carotene (8.61–11.51 µg g^{-1}) and the rest nine inbreds had less β-carotene (0.41–2.77 µg g^{-1}), despite having the favourable allele. Of the 385 inbreds screened, only five inbreds viz. HP465-19, HP465-27, HP467-3, HP467-15 and HP467-20 had the favourable allele of both *lcyE* and *crtRB1* genes.

4. Discussion

The present study analysed the presence of allelic diversity of *lcyE* (in 5′TE region) and *crtRB1* (in 3′TE region) among diverse set of maize inbreds. In the present study, 3.38% of the inbreds had the

Table 2. List of inbreds identified with the favourable allele (3′TE polymorphism) of *crtRB1* gene (543 bp)

S. No.	Genotype	Pedigree	Source
1	SE547	Ludhiana semi-exotic heterotic pool	PAU, Ludhiana
2	Pant120	[SPMAT/EV89MDREY]-51-2-B-1-6-B-2-B-B-B-B	GBPUAT, Pantnagar
3	Pant125	Pob446-74-2-3-B-B-B-2	GBPUAT, Pantnagar
4	V372	PRO-337OP⊗-6-4-1-3-4-1-#-⊗b-##-⊗b	VPKAS, Almora
5	MGUDM-RIL47	(NAI116 × CM139)-⊗-⊗-⊗-⊗- ⊗	IARI, New Delhi
6	KDMI4	X1(Y) Pool	UAS, Bangalore
7	EI116	X2 W 3997	MPUAT, Udaipur
8	HP233-20	[DTPYC9-F11-2-3-1-1-B-B × DTPYC9-F46-1-2-1-1-B]-B-1-1-B-B	CIMMYT-HarvestPlus
9	MGUQPM-MAS4979	(CM150 × CML161)-BC1-BC2-⊗-⊗-⊗-⊗	IARI, New Delhi
10	BAJIM 06-10	HAREC Pool95-171-1-5-3-7-2	CSK-HPKV, Bajaura
11	HP465-19	(KUI carotenoid syn-FS11-1-1-B-B-B/(KU1409/DE3/KU1409)S2-18-2-B)-B-2	CIMMYT-HarvestPlus
12	HP465-27	(KUI carotenoid syn-FS17-3-2-B-B-B/(KU1409/DE3/KU1409)S2-18-2-B)-B-1	CIMMYT-HarvestPlus
13	HP467-3	(KUI carotenoid syn-FS11-1-1-B-B-B/(KU1409/DE3/KU1409)S2-18-2-B)-B-2	CIMMYT-HarvestPlus
14	HP467-15	(KUI carotenoid syn-FS11-1-1-B-B-B/(KU1409/DE3/KU1409)S2-18-2-B)-B-3	CIMMYT-HarvestPlus
15	HP467-20	(KUI carotenoid syn-FS17-3-2-B-B-B/(KU1409/DE3/KU1409)S2-18-2-B)-B-1	CIMMYT-HarvestPlus

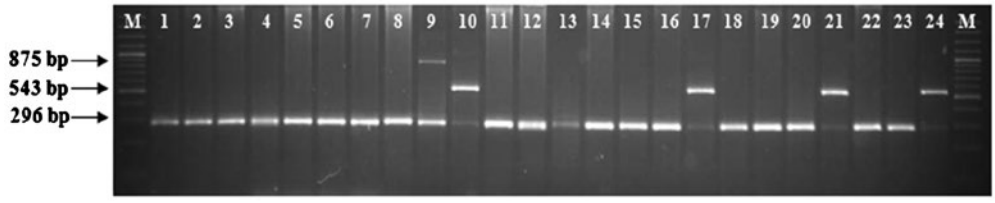

Figure 2. Allelic variations at 3′TE region of *crtRB1* gene.

Note: Lane 10, 17, 21 and 24: Inbreds with the favourable allele (*allele 1*: 543 bp amplicon); Lane 1–8, 11–16, 18–20, 22 and 23: Inbreds with the unfavourable allele (*allele 3*: 296 bp amplicon); Lane 9: Inbred with the unfavourable allele (*allele 2*: 875 bp + 296 bp amplicons); M: 100 bp DNA ladder.

favourable allele (*allele 4*) at *lcyE* locus, while 96.62% inbreds possessed the unfavourable allele (*allele 2*) (Table 1). This is in congruence with the earlier report on the existence of the favourable allele in extremely less frequency (Harjes et al., 2008).

Though *allele 4* is having the favourable effect, for most of the inbreds identified except for the new set of CIMMYT-HarvestPlus inbreds, the β-carotene in the endosperm was at par with the inbreds possessing the unfavourable allele (*allele 2*). This could be due to various reasons; firstly, carotenoid biosynthesis pathway is regulated by many genes, the identified inbreds may not be having the favourable alleles of other key genes governing enhancement of β-carotene in the pathway. This is well evident from the results of the present study that none of the eight inbreds (having low β-carotene) had the favourable allele of *crtRB1*, the other crucial gene responsible for higher accumulation of β-carotene. Even though the favourable *lcyE* allele diverts the pathway towards the β-branch, most of them will get hydroxylated to non-provitamin A carotenoids, like zeaxanthin, downstream in the pathway leading to drastic reduction of β-carotene in the endosperm (Vallabhaneni et al., 2009). In contrast, the new set of CIMMYT-HarvestPlus inbreds also had the favourable allele of *crtRB1* gene and hence high β-carotene. Another interesting fact emerged was that H16, a white endosperm line showed the presence of *lcyE* 5′TE favourable allele. This inbred lacks the dominant allele for *phytoene synthase* (*PSY1* or *Y1*), the gene which is responsible for the first committed step in the pathway. The dominant *Y1* gene coverts geranylgeranyl diphosphate to phytoene and is responsible for the yellow colouration of the maize endosperm (Buckner et al., 1990). This phytoene serves as a substrate for further biochemical conversions in the pathway by the

other downstream genes. Since the inbred H16 lacks the functional enzyme for this basic conversion step in the pathway, there may not be any substrate available for *lcyE* to act upon to divert them to β-branch of the pathway. Secondly, effect of the background genome in which this allele is present may also play a role in the regulation of carotenoid biosynthesis pathway. The favourable *lcyE* allele was originally identified in the temperate maize germplasm through association mapping approach (Harjes et al., 2008), whereas these alleles may not be effective in the genetic background of tropical and subtropical Indian maize inbreds. Babu et al. (2013) also found similar results when they validated the effect of *lcyE* 5′TE allele across five populations. Thirdly, though the allele is amplifying 650 bp amplicon, there could also be some nucleotide polymorphisms present within the *lcyE* 5′TE region among the identified inbreds, leading to the variation in the β-carotene concentration.

The inbreds panel was also characterized for allelic variation in *crtRB1* using gene-specific markers. Among the inbreds screened in the present study, 3.90% of the inbreds revealed the favourable allele (Figure 2), while rest (96.10%) showed unfavourable alleles. The presence of the favourable allele in extremely less frequency in population supports earlier findings of Yan et al. (2010).

CrtRB1 is a hydroxylase gene that causes hydroxylation of β-carotene to non-provitamin A carotenoids such as zeaxanthin. The favourable allele (543 bp) of *crtRB1* gene causes reduced transcript expression, thereby blocking the conversion of β-carotene to zeaxanthin that leads to the higher accumulation of β-carotene in the maize kernel (Yan et al., 2010). However, among the identified inbreds having the favourable allele of *crtRB1* 3′TE gene, except for the new set of CIMMYT-HarvestPlus inbreds, other inbreds did not record high β-carotene concentration (0.41–2.77 μg g^{-1}). The new set of CIMMYT-HarvestPlus genotypes having the favourable allele of *crtRB1* 3′TE gene showed a mean kernel β-carotene concentration of 10.1 μg g^{-1} (8.61–11.51 μg g^{-1}).

The contrast in kernel β-carotene concentration could be attributed to the influence of genetic background in which they are present leading to difference in the phenotypic expression. This could also be due to the presence/absence of favourable alleles of other important genes in the carotenoid biosynthesis pathway. Inbreds (with favourable allele) with low β-carotene did not have the favourable allele of *lcyE* gene. Further to the results of *lcyE* gene, a white endosperm line EI116 showed favourable *crtRB1* 3′TE allele, but was devoid of any carotenoids. There could also be a possibility of nucleotide polymorphisms present in the *crtRB1* gene that may cause difference in expression of the gene causing phenotypic variations (Vignesh, Nepolean, Hossain, Singh, & Gupta, 2013). This can be validated by comparing nucleotide difference vis-à-vis estimating the accumulation of transcript in the contrasting genotypes.

β-carotene-rich inbreds with favourable allele(s) of both *lcyE* and *crtRB1* can be utilized in the breeding programme through many ways. Firstly, to develop provitamin A-rich maize hybrids, the new set of CIMMYT-HarvestPlus inbreds with high β-carotene and favourable alleles can be used as donors in the breeding programme (Vignesh et al., 2012). Since the effects of both genes are additive in nature (Yan et al., 2010), MAS can effectively introgress the favourable alleles of both *crtRB1* and *lcyE* in diverse genetic backgrounds. Babu et al. (2013) reported that the favourable *crtRB1* allele is twice efficient in accumulating higher β-carotene when present in homozygous condition than under heterozygous condition. Thus, to develop hybrids homozygous for *crtRB1* favourable allele, both the parents should be introgressed with the favourable allele. Secondly, newly developed CIMMYT-HarvestPlus inbreds (having high β-carotene) possess limited genetic diversity. Hence, these can be crossed with inbreds of Indian origin (having low β-carotene) to generate hybrids with moderate level of β-carotene. Thirdly, the favourable allele can be introgressed into the parents of already released hybrids through MAS to exploit the established grain heterosis and adaptability. Exploiting this strategy, two each of extra early and medium maturity hybrids have been improved for kernel β-carotene (Muthusamy et al., 2014).

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