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Genetic variability for kernel β-carotene and utilization of *crtRB1* 3'TE gene for biofortification in maize (*Zea mays* L.)

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Abstract

Carotenoids are the major sources of dietary precursor of vitamin A and act as potential antioxidant besides preventing diseases such as night blindness in humans. Vitamin A deficiency is a global problem, but is particularly prevalent in developing countries like India, where 31 percent of pre-school children are reportedly affected. Evaluation of genetic variability for kernel β-carotene in 105 maize inbreds of diverse pedigree from India- and CIMMYT- origin revealed significant variation ranging from 0.02 to 16.50 µg/g. One of the key reasons for wide variation of kernel β-carotene is due to the allelic variation at *crtRB1* 3'TE gene. Five among 95 inbreds possessed the favourable *crtRB1* 3'TE allele with a mean β-carotene concentration of 0.86 µg/g. In contrast, the same allele detected in 20 CIMMYT-Maize HarvestPlus genotypes contributed a mean kernel β-carotene concentration of 11.29 µg/g. The contrast in β-carotene concentration in Indian and CIMMYT genotypes could be attributed to the presence of SNPs and InDels in *crtRB1* 3'TE locus, along with the presence/absence of favourable alleles of other important genes influencing the carotenoid biosynthetic pathway. Marker-assisted breeding has been initiated, to introgress the *crtRB1* 3'TE favourable allele using high β-carotene CIMMYT inbreds as donors, to develop provitamin A-rich maize cultivars suitable for maize growing regions in India.

Key words: Maize, β-carotene, carotenoid biosynthesis, genetic variability, biofortification

Introduction

Vitamin A or retinol is an essential nutrient needed by humans for the normal functioning of the visual system, growth and development, and maintenance of epithelial cell integrity, immune system and reproduction [1, 2]. Humans and animals are unable to synthesize their own vitamin A requirement, which needs to be therefore provided through dietary provitamin A carotenoids. Vitamin A deficiency (VAD) alone affects over 250 million pre-school children, and accounts for about 70% of the childhood deaths worldwide (http://www.who.int/nutrition/topics/vad/en). World Health Organization (WHO) estimates that 250,000 to 500,000 children become blind every year due to VAD and it further contributes to predisposition of several major diseases, such as anemia, diarrhea, measles, malaria and respiratory infections [3-6]. Young children, pregnant women and lactating mothers are most vulnerable to VAD as it also contributes to maternal death, poor pregnancy and lactation [7, 8]. Thus, efforts directed to minimize VAD have a positive impact on the health of human beings particularly, women and children.

Development of micronutrient-enriched staple plant foods through plant breeding, a process referred to as "biofortification" holds significant promise for sustainable food-based solutions to the hidden hunger [9-11]. Biofortification is more sustainable and cost-efficient compared with the traditional nutrition interventions such as supplementation, food fortification, and dietary diversification [11, 12]. Maize is one of the most important food crops in the world and, together with rice and wheat, provides at least 30% of the food calories to more than 4.5 billion people in 94 developing countries [13]. As compared to rice and wheat, only the yellow kernel type maize possess significant levels of provitamin A carotenoids among the cereals [14]. Maize is also a model cereal crop for developing strategies to solve global micronutrient deficiencies and shows promise for provitamin A biofortification especially through molecular marker-assisted breeding [15, 16]. All yellow genotypes of maize contains carotenoids, although only a few components of carotenoids (β-

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carotene, α-carotene and β-cryptoxanthin) possess provitamin A activity, while a predominant fraction consists of non-provitamin A carotenoids *viz*., zeaxanthin and lutein [17].

Plant breeding has been the main stay of programmes to enhance staple food crops with sufficient levels of micronutrients such as provitamin A carotenoids to meet the needs of populations at risk [18, 19]. The first step in breeding maize for enhanced β-carotene involves an assessment of genetic variability existing in adapted germplasm for developing an appropriate breeding strategy. A few studies have found significant genetic variation for kernel β-carotene in yellow maize inbreds and hybrids [20-22]. However, limited reports have been published on the range of variation of β-carotene in diverse tropical-adapted Indian maize inbred lines.

Recent advances in molecular biology led to the clear understanding of genes involved in the carotenoid biosynthetic pathway and identification of molecular markers for favourable alleles at key candidate genes influencing β-carotene concentration [23-25]. Yan *et al.* [25] confirmed that *crtRB1* (*β-carotene hydroxylase*) is an important gene associated with the β-carotene concentration in maize kernels. Through association mapping approach, three polymorphisms *viz*., 5'TE (in the 5'- Untranslated Region), InDel4 (in the coding region) and 3'TE (spanning the sixth exon and 3'-Untranslated Region), were identified for *crtRB1* gene that were significantly associated with variation for the target trait [25]. PCR-based co-dominant markers were identified for all the three *crtRB1* polymorphisms [25] paving the way for rapid improvement of provitamin A status in maize kernels through marker-assisted selection (MAS) [16]. The *crtRB1* 3'TE favourable allele (*allele 1*, 543 bp) associated with reduced transcript expression of the gene correlate with higher β-carotene concentrations, with an average increase of 6.50 µg/g β-carotene in maize endosperm above the unfavourable allelic class [25]. Hence, screening of Indian maize germplasm to identify inbreds with *crtRB1* 3'TE favourable allele is important in breeding provitamin A enriched maize.

Therefore, the present study was undertaken with the following objectives: (i) to assess the genetic variability for kernel β-carotene in a selected panel of maize inbred lines (developed in Indian and CIMMYT maize breeding programmes) for identification of promising inbreds for maize biofortification; and (ii) to assess the panel of inbreds for presence of favourable *crtRB1* 3'TE allele influencing provitamin A concentration in maize.

Materials and methods

Plant materials

A diverse panel of 95 inbred lines from India and CIMMYT showing significant variation for kernel colour (ranging from pale yellow to deep orange) were selected for the study. The panel included; (i) 21 inbreds developed at Maize Genetics Unit, Indian Agricultural Research Institute (IARI), New Delhi; (ii) six inbreds from Vivekananda Parvatiya Krishi Anusandhan Sansthan (VPKAS), Almora; (iii) four inbreds from Punjab Agricultural University (PAU), Ludhiana; (iv) four quality protein maize (QPM) inbreds developed by Directorate of Maize Research (DMR), New Delhi; (v) four inbred lines developed at CSK-HPKV, Hill Agricultural Research and Extension Centre (HAREC), Bajaura; (vi) four inbreds from CCS-Haryana Agricultural University (CCS-HAU), Karnal; (vii) two inbreds from UAS-Nagenahalli Research Centre, Karnataka; (viii) 10 inbred lines developed under the All India Coordinated Maize Improvement Project (AICMIP); (ix) six inbreds developed by CIMMYT, Mexico and (x) 34 inbred lines developed under the CIMMYT-Maize HarvestPlus program, Mexico. All these inbred lines were evaluated at three locations *viz*., (i) IARI Experimental Farm, New Delhi, (ii) VPKAS, Almora and (iii) CSK-HPKV, Bajaura, during *Kharif* 2010. Besides a new set of 20 inbred lines (with *crtRB1* 3'TE favourable allele) developed under the CIMMYT-Maize HarvestPlus programme were also evaluated during the same season at IARI Experimental Farm. Recommended cultural practices were followed to raise a good crop and each entry was carefully self-pollinated to avoid any possible contamination from foreign pollen. Self-pollinated ears from each entry were harvested separately; seeds were shelled under shade and stored in darkness at 4°C until carotenoid extraction.

Carotenoid extraction and quantification of β-carotene

Seeds samples were ground to fine powder and further extraction steps were carried out under dark conditions as carotenoids undergo photo-oxidation in presence of light [26, 27]. Carotenoid extraction from maize seeds was carried out using the procedure described by Kurilich and Juvik [28] with some minor modifications. Quantification of the β-carotene was carried out using a Water Alliance HPLC System (Waters Chromatography, Milford, MA). Samples were eluted through YMC Carotenoid C30 column (5 µm, 4.6 × 250

mm; YMC, Waters) and detected with a photodiode array detector (PDA). The mobile phase consisted of methanol: tert-butyl methyl ether (80:20, v/v), and the flow rate was 1 ml min^{-1}. Six dilutions of β-carotene standard (SIGMA chemicals) were used to make the standard curve for β-carotene and the concentration of β-carotene in each inbred was measured by standard regression with external standards. To maximize detection of β-carotene, absorbance was measured at 450 nm.

Isolation of DNA and PCR amplification of the crtRB1 3'TE gene

Genomic DNA was isolated from 3 weeks old young seedlings of each of the inbreds using standard CTAB procedure with minor modifications. Polymerase chain reaction was performed using *crtRB1* 3'TE gene-specific primers and PCR amplification was carried out using the standard cycle conditions [25]. Along with the 95 selected inbreds, 20 CIMMYT inbreds with *crtRB1* 3'TE favourable allele were also used [25]. Amplified fragments were separated using 1.5% agarose gel based electrophoresis and were scored for the presence of favourable alleles.

Results and discussion

Genetic variation for kernel β-carotene concentration

The study revealed significant variation for kernel β-carotene concentration with a range of 0.02 to 16.50 μg/g across Indian and CIMMYT genotypes. However, the kernel β-carotene concentration among the selected 95 inbreds ranged from 0.02 to 1.75 μg/g across three locations. Egessel *et al*. [20] reported a range of 0.5 to 3.4 μg/g (mean; 1.5 μg/g) β-carotene concentration among a set of maize hybrids. A range of 0.7 to 4.7 μg/g across four trials with a mean of 1.9 μg/g of kernel β-carotene was reported by Menkir *et al*. [21] while evaluating a group of tropical yellow maize inbreds. Chander *et al.* [22] reported that kernel β-carotene varied from 0.01 to 1.72 μg/g in a set of inbreds from the Chinese maize breeding programme.

On the other hand, a much broader range of 6.50 to 16.50 μg/g kernel β-carotene was observed in the 20 newly developed CIMMYT-Maize HarvestPlus inbreds. Three inbreds, HP 465-26 (16.50 μg/g), HP 465-41 (15.90 μg/g) and HP 467-22 (15.19 μg/g) were found to be highly promising with >15 μg/g of β-carotene. Other promising inbreds include HP 465-30 (13.93 μg/g), HP 467-59 (12.91 μg/g), HP 465-20 (12.36 μg/g) and HP 467-23 (12.26 μg/g). Among these set of genotypes, eleven inbreds were found to have β-carotene in the range of >10.00 to 15.00 μg/g, while six inbreds recorded kernel β-carotene between 6.50 to 10.00 μg/g (Fig. 1). Selected maize genotypes with high kernel β-carotene concentrations are presented in Table 1.

Among the 95 inbred lines, QPM-MAS-4979 was found to have the highest kernel β-carotene concentration of 1.75 μg/g across three locations. Other genotypes having >1 μg/g of kernel β-carotene include HPLET08-Entry40 (1.27 μg/g) and HPLET08-Entry20 (1.18 μg/g). All the other genotypes had less than 1 μg/g of kernel β-carotene (Fig. 1).

The study has shown significant variation for kernel β-carotene among the sister lines. The eight QPM-MAS genotypes derived from CM150, showed

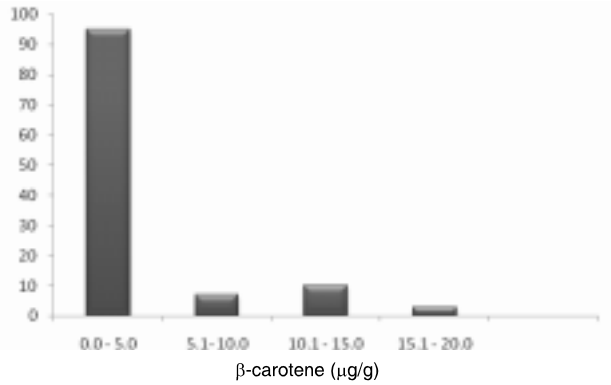

Fig. 1. Variation for kernel β-carotene concentration among the selected genotypes

Table 1. Promising inbreds with high β-carotene concentration identified in the study

S.No.	Inbred lines	Source	β-carotene* (μg/g)
1	HP 465-26	CIMMYT-Maize HarvestPlus	16.50
2	HP 465-41	CIMMYT-Maize HarvestPlus	15.90
3	HP 467-22	CIMMYT-Maize HarvestPlus	15.19
4	HP 465-30	CIMMYT-Maize HarvestPlus	13.93
5	HP 467-59	CIMMYT-Maize HarvestPlus	12.91
6	HP 465-20	CIMMYT-Maize HarvestPlus	12.36
7	HP 467-23	CIMMYT-Maize HarvestPlus	12.26
8	HP 467-5	CIMMYT-Maize HarvestPlus	11.97
9	HP 465-33	CIMMYT-Maize HarvestPlus	11.88
10	HP 467-9	CIMMYT-Maize HarvestPlus	11.57

*Value is based on IARI, New Delhi data (*kharif* 2010)

wide β-carotene variation from 0.20 to 1.75 μg/g. Besides, four DM-RIL genotypes showed 0.09 to 0.80 μg/g of kernel β-carotene. Even though the inbred lines in each of the above group were developed from a particular source population, they exhibited variability for kernel β-carotene. The variation for kernel β-carotene among the sister lines may be due to the difference in segregation and fixation of different gene/allele combinations among the genotypes developed from the same source population. Similar trend of variation was reported earlier for total carotenoid in maize, among the DMRQPM sister lines [29] and in a $F_{6/7}$ RIL population developed by By804 × B73 [30].

The study also revealed a similar trend of variation for β-carotene among the QPM genotypes (0.11 to 1.75 μg/g) as compared to the non-QPM genotypes (0.02 to 1.27 μg/g). This indicates that the QPM trait has no discernible influence on kernel β-carotene concentration in maize. Relatively similar variation of kernel β-carotene among the QPM and non-QPM genotypes shown in the present study is in congruence with an earlier study on total carotenoid variation in maize [29].

The Maize HarvestPlus program has set a target level of 15 μg/g of β-carotene in maize kernel to meet the recommended dietary allowance for humans to overcome VAD (www.harvestplus.org). The concentration of β-carotene (0.02 to 1.75 μg/g) among 95 inbreds was significantly low to meet the above stated requirement. Interestingly, β-carotene concentration among the new set of 20 inbred lines (developed under the CIMMYT-Maize HarvestPlus Programme) was found to be high and has the potential to meet the target level set by HarvestPlus programme. These promising inbreds therefore, can be utilized as donors in breeding programme to meet the dietary requirement.

Screening for favourable allele at crtRB1 3'TE gene

The 95 inbreds used in the study were also characterized for *crtRB1* 3'TE gene using gene-specific markers. The 3'TE polymorphism of *crtRB1* gene that spans the 6th exon and the 3'-UTR has three alleles *viz*., *allele 1* (543 bp; without TE insertion), *allele 2* (296 + 875 bp; with 325 bp insertion) and *allele 3* (296 + 1221 + 1880 bp; with 1250 bp insertion) that were associated with variation in β-carotene accumulation in the kernel. *Allele 1* is known as a favourable allele for enhancing the β-carotene concentration by reducing transcript expression of the *crtRB1* gene, whereas *allele 2* and *allele 3* cause unfavourable effects. Among the 95 inbreds screened, only 5 inbreds *viz*., DM-RIL47, QPM-MAS-4972, QPM-MAS-4974, QPM-MAS-4979 and HPLET08-Entry10 revealed the favourable allele (Fig. 2), while rest showed unfavourable alleles. However, the inbreds having favourable allele of *crtRB1* 3'TE gene did not record high β-carotene concentration across the three locations; DM-RIL47 recorded 0.80 μg/g; HPLET08-Entry10 had 0.41 μg/g; while QPM-MAS-4972, QPM-MAS-4974 and QPM-MAS-4979 showed 0.89 μg/g, 0.43 μg/g and 1.75 μg/g, respectively. The effect of favourable allele of *crtRB1* 3'TE gene was compared with 20 new inbred lines developed under the CIMMYT-Maize HarvestPlus Programme [25]. These genotypes having the favourable allele of *crtRB1* 3'TE gene contributed a mean kernel β-carotene concentration of 11.29 μg/g. The contrast of variation in kernel β-carotene between the above mentioned five genotypes and newly developed genotypes despite the presence of *crtRB1* 3'TE favourable allele, could be attributed to the presence of nucleotide polymorphisms such as SNPs/ InDels in the 3'TE region of *crtRB1* gene, along with the presence/absence of favourable alleles of other important genes in the carotenoid biosynthetic pathway.

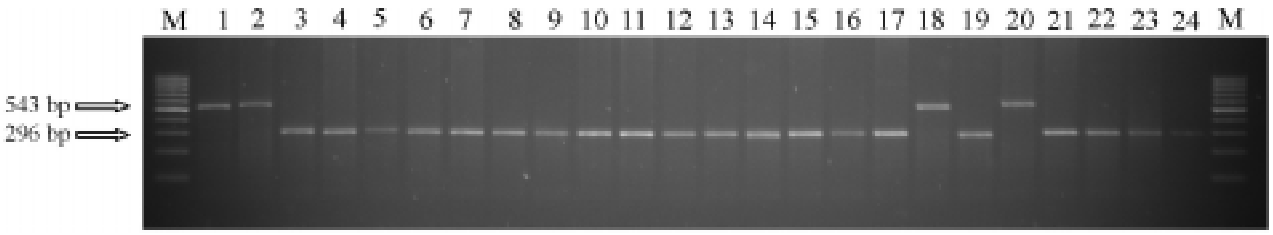

Fig. 2. Allelic variation at *crtRB1* 3'TE gene among the Indian and CIMMYT genotypes. Lane 1 - HP 465-26; Lane 2 - HP 467-22 (Inbreds from CIMMYT-Maize HarvestPlus Programme having high β-carotene and *crtRB1* 3'TE favourable allele: 543 bp); Lane 18 - DM-RIL47 and Lane 20 - QPM-MAS-4979 (Indian inbreds with favourable allele: 543 bp); Lane 3 to 17, 19, 21 to 24 - Indian inbreds with unfavourable allele (296 bp); M: 100 bp DNA ladder

Marker-assisted selection towards enrichment of β-carotene

The β-carotene concentration in the Indian genotypes was found low and the inbreds identified with *crtRB1* 3'TE favourable allele also had less β-carotene. However, the CIMMYT-HarvestPlus bred maize inbreds with high kernel β-carotene and *crtRB1* 3'TE favourable allele [24] offer tremendous possibility to breed for high β-carotene maize through MAS. Using these β-carotene enriched genotype as donors, marker assisted backcross breeding programme has been already initiated at Maize Genetics Unit, IARI. The *crtRB1* 3'TE favourable allele from these high β-carotene genotypes is being introgressed into elite Indian inbreds to reach the HarvestPlus's target level of 15 µg/g of kernel β-carotene in the Indian maize hybrids.

The present investigation revealed the presence of genetic variation for kernel β-carotene in the selected maize inbreds, leading to the identification of five genotypes having *crtRB1* 3'TE favourable allele. The availability of newly developed inbreds with high β-carotene and *crtRB1* 3'TE favourable allele (developed under the CIMMYT-Maize HarvestPlus Programme) offer tremendous potential for further developing provitamin A-enriched maize cultivars through marker-assisted backcrossing in genetic backgrounds adapted to India.

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