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Phenotyping of maize inbred lines for beta-carotene and determining relationship with total carotenoids and kernel colour in maize

Rashmi, Mahak Tufchi[1], Mrinal Shastry and N. K. Singh*

Department of Genetics and Plant Breeding, G. B. Pant University of Agriculture and Technology, Pantnagar 263 145, Uttarakhand; [1]Directorate of Maize Research Unit, PAU, Ludhiana 141 004

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Abstract

Availability of variable amount of provitamin A carotenoids in maize (*Zea mays* L.) germplasm makes it a potential crop that can be exploited in biofortification programs. However, characterization of germplasm for identification of high provitamin A might help maize breeder to integrate high provitamin A lines with other important parameters mainly yield. The present study was, therefore, undertaken to determine total carotenoids (TC) and beta-carotene (βC) in high yielding 32 inbred lines of maize and also to develop simple selection criteria based on relationship of βC with TC and kernel colour. The 32 inbred lines exhibited βC of minimum of 0 µg/g (white kernel lines) to 4.814 µg/g (Pant13k 2121) dry weight with mean value of 2.58 µg/g whereas TC was found to vary from minimum 3.34 µg/g to maximum of 27.44 µg/g dry weight with mean value of 19.28 µg/g. TC and βC was relatively higher for orange shades as compared to different yellow shades. Kernel color established positive correlation with TC as well as βC with very low coefficient of determination (0.108). This indicates that visual selection based on kernel color may not be effective improving carotenoids in maize. However, association of TC and βC was significantly positive with high coefficient of determination.Further, molecular characterization is done by *LcyE* and *CrtRB*1 markers.

Key words: Maize, *LcyE, CrtRB1* beta carotene, total carotenoids

Introduction

Carotenoids are the large class of yellow, red and orange pigments derived from isoprenoids which are produced by plants and furthermore, dietery essentials to animals as they provide nutrtional value as provitamin A and non provitamin A compounds. Provitamin A are precursors of vitamin A, which is an important micronutrient for human health [1]. Vitamin A deficiency (VAD) can causes various disease in human body like xerophthalmia, infant morbidity and mortality, and depressed immunological responses [2]. Maize (*Zea mays* L.) can naturally accumulate both provitamin A and non provitamin A carotenoids in its kernel, and is known for its genetic diversity of carotenoids content and profiles [3, 4]. However, provitamin A usually constitutes only 10 to 20% of total carotenoids in maize kernel and the commonly cultivated and consumed yellow maize cultivars have less than 2 µg/g dry weight provitamin A. Exploitation of the natural genetic diversity of maize in carotenoids through biofortification by combining conventional and molecular breeding can increase provitamin A concentration in maize endosperm [4, 5]. Biofortification offers safe, effective, cheap and sustainable approach to combating vitamin A and other micronutrient deficiencies [6]. However, characterization of germplasm is necessary by using morphological as well as molecular parameters so that potential lines could be identified for biofortification for beta carotene in maize.

Due to advances in molecular biology, the genes encoding enzymes involved in carotenoid biosynthetic pathway are studied [7, 8] and furthermore, molecular markers for favourable alleles at key candidate genes have also been characterized and shown to contribute significantly to accumulation of provitamin A and total carotenoid in maize kernels [9, 10]. Four polymorphic sites in the gene encoding *licopene epsilon cylase* (*LcyE)* which were associated with the variation in ratio of carotenoids in the alpha and beta branches of the carotenoid biosynthetic pathway, leading to threefold

*Corresponding author's e-mail: narendraksingh2@gmail.com
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increase in provitamin A [9, 10]. Whereas three polymorphic sites in another downstream gene encoding *beta carotene hydroxylase* (*CrtRB1*) enzyme accounting for 40% of the observed variation in beta carotene content were identified in maize kernels. The present study was, therefore, carried out to estimate total and beta carotene contents in inbred lines of maize, to study the relationship between kernel color with total carotenoids and beta carotene content, relationship between total carotenoids and beta carotene content and to analys allelic diversity at LcyE and CrtRB1 loci using DNA based functional markers.

Materials and methods

The thirty two promising inbred lines used for the study which showing significant variation for kernel color. The lines includes local varieties, DMR (Directorate of Maize Research) accessions and CIMMYT accessions. Recommended cultural practices were followed to raise a good crop during *kharif* 2013 and each entry was carefully self pollinated to avoid any possible contamination from foreign pollen. Self pollinated ears from each entry were harvested separately. The harveasted ears were dried in shade and stored in cool place away from sunlight. For total carotenoids and beta carotene extraction, seeds shelled out of ears and grinded to fine flour and stored at –20°C till the extraction is done. All the procedures were commenced in dark conditions.Carotenoids extraction proctocol developed by Torbert Rochefords Lab was used for the extraction of carotenoids from maize kernels (http://www.cropsci.uiuc.edu/faculty/rocheford/quick_carotenoid_analysis_protocol.pdf). Spectrophotometer was used to record optical density values of each sample at 450 nm and subsequently carotenoids concentration was determined using Lambert-Beer equation [11]. The beta-carotene content estimated by the procedure proposed earlier [12]. The Association: St. Paul, MN. The six dilutions of standard beta carotene (SIGMA) were used to make standard curve for beta-carotene and the concentration of beta-carotene in each inbred line was measured from this curve. DNA was isolated from three weeks old young seedlings of each of inbred lines using standard CTAB method with minor modifications. DNA extracted was purified by PCI and RNase treatments. DNA quantificaion done by measuring absorbance of DNA sample using Eppendorf BioPhotometer Plus. Polymerase chain reaction caried out by using LcyE (LcyE 5'TE, LcyE-SNP (216), LcyE 3'TE) and CrtRB1 (CrtRB1 5'TE and CrtRB1 3'TE) gene specific primers and cycle conditions (denaturation at 94°C for 1 min; annaealing at 59 to 62°C depending on specific primer for 1 min followed by extension period for 1 min at 72°C. The reactions were subsequently subjected to 34 additional cycles after reaching the final annealing temperatures. This was followed by a final extension at 72°C for 5 min. The amplified PCR products were resolved on 3.5% Metaphor gel.

Results and discussion

The present study revealed significant variation for total carotenoids in maize inbred lines which vary from minimum 3.34 μg/g to maximum of 27.44 μg/g dry weight with mean value of 19.28 μg/g. Among three major cereal crops, maize is the only crop that shows appreciable amount of total carotenoids content in its kernel [5]. Due to its triploid nature of endosperm; dosage effect plays important role in carotenoids accumulation [13, 14]. Wide range of variability for carotenoids (5.5 μg/g to 66 μg/g) with mean value of 23 μg/g was also observed [10]. Allelic variations and dosage effect may be responsible for wide range of variability for carotenoids in yellow maize [15]. Significant variation for kernel carotenoids in established maize inbred lines has been earlier reported [16-18].

The significant variation for beta-carotene content also revealed by present study where it varies from 0 μg/g to 4.814 μg/g dry weight with mean value of 2.58 μg/g. Egessel *et al*. [13] reported beta-carotene concentration of 0.5 μg/g to 3.4 μg/g with mean value of 1.5 μg/g among set of maize hybrids. A range of 0.7 μg/g to 4.7 μg/g across four trials with mean of 1.9 μg/g of kernel beta-carotene was reported by Menkir *et al.,* (2008) while evaluating a group of tropical yellow maize inbreds. Vignesh *et al.,* [19] reported variation for beta-carotene with range of 0.02 to 16.50 μg/g across indian and CIMMYT-HarvestPlus genotypes.

All the thirty two lines were carefully analyzed for kernel color with the help of Color Fan 1 of the Royal Horticultural Society and differentiated into different shades of orange and yellow. The lines were classified into 17 different groups (G1 to G17) and aligned along with total carotenoids and beta-carotene content (Table 1). When correlation between total carotenoids and beta-carotene content were studied, it was found that correlation between these observed to be positive and significant (Fig. 1). Coefficient of determination (R^2) was noted to be high which revealed that about 67 percent of variance in beta-carotene content could be determined by total carotenoids. Results of the present study further signified that lines selected based on high

Table 1. Kernel colour group, total and beta-carotenoids of maize inbred lines

Groups	Colour	Inbreds	Total carotenoids (µg/g)	Beta-carotene (µg/g)
G1	Yellow Orange11A	Pant13k 2126	16.40	2.901
G2	Yellow Orange14A	Pant13k 2124	14.92	1.975
G3	Yellow Orange17A	Pant13k 2110	12.69	1.481
		Pant13k 2101	15.00	1.604
G4	Yellow Orange21A	Pant13k 2093	12.00	2.098
G5	Yellow Orange23A	Pant13k 2114	16.89	1.728
		Pant13k 2099	21.69	3.518
		Pant13k 2096	21.61	2.592
		Pant13k 2094	20.75	3.024
		Pant13k 2098	19.03	2.160
G6	Yellow Orange B	Pant13k 2115	17.15	2.222
G7	Yellow Orange17B	Pant13k 2125	14.00	1.049
G8	Yellow Orange21B	Pant13k 2092	18.69	2.654
		Pant13k 2120	15.52	1.851
G9	Yellow Orange23B	Pant13k 2106	16.89	1.790
G10	Orange23A	Pant13k 2113	27.44	2.76
G11	Orange24A	Pant13k 2118	23.92	2.839
G12	Orange25A	Pant13k 2100	21.26	3.148
		Pant13k 2119	22.81	3.271
G13	Orange28A	Pant13k 2117	27.10	3.20
G14	Orange25B	Pant13k 2102	16.03	2.037
G15	OrangeN25C	Pant13k 2116	18.01	2.716
		Pant13k 2103	18.95	2.037
		Pant13k 2108	22.72	4.259
		Pant13k 2121	24.01	4.814
		Pant13k 2105	20.24	1.728
G16	OrangeN25D	Pant13k 2128	19.55	3.024
		Pant13k 2109	23.32	3.024
		Pant13k 2130	20.92	2.469
		Pant13k 2112	27.18	4.259
		Pant13k 2111	26.84	4.135
G17	White	Pant13k 2132	3.34	0

total carotenoids may also be high in beta carotenoids. Thus, selection criteria based on total carotenoids can be used for screening of large number of germplasm or breeding population since determination of total carotenoids using Quick carotenoids extraction method is quick and less expensive. The selected lines can further be analyzed in detail using different chromatographic approaches for selecting few promising lines with high beta-carotene content.

When relationship between total carotenoids and beta-carotene content with kernel color was investigated, it was found that mean value for total carotenoids and beta-carotene content was relatively higher for orange shades as compared to different yellow shades. Correlation studies between kernel color groups and total carotenoids were determined to find out the nature and amount of association between these two parameters (Fig. 2). An assumption was initially made

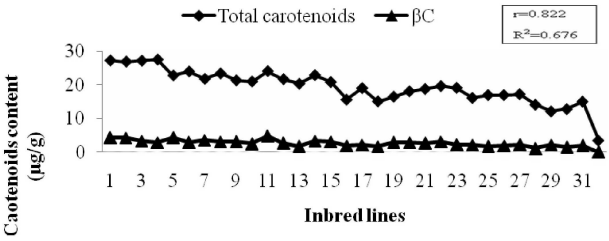

Fig. 1. Relationship between total carotenoids and beta-carotene in lines

that a significant positive association with high coefficient of determination could be use in selection of lines with high carotenoids using simple criteria of kernel color. The study revealed that kernel color is positively and significantly correlated (0.329) with total carotenoids with very low coefficient of determination (0.108). This indicates that kernel color cannot be used reliably in selection of lines with higher carotenoids. Positive correlation between kernel color and carotenoids along with low coefficient of determination were also reported earlier by [8, 6, 18, 20]. Beta-carotene was also assessed for its association with kernel color (Fig. 3). Analysis revealed positive association (0.287) with low coefficient of determination (0.082). Thus, the present investigation with 32 inbred lines did not indicate any strong role of kernel color in selection of lines with high beta-carotene. Hirschberg [8] made comparisons

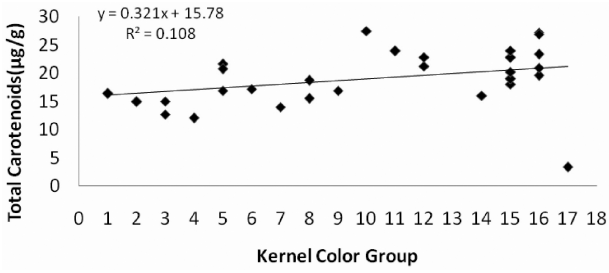

Fig. 2. Correlation between kernel carotenoids and different colour groups

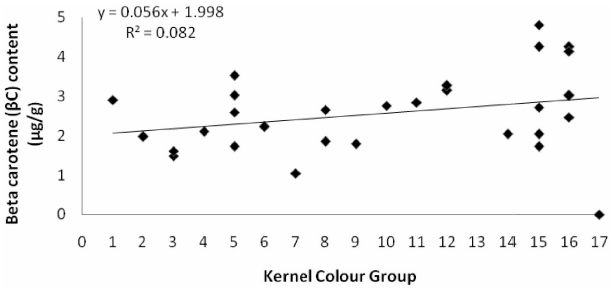

Fig. 3. Correlation between different colour groups with beta-carotene content

Table 2. Pedigree of maize inbred lines

S.No.	Inbred lines	Pedigree
1	Pant13k 2092	Pop 31⊗18-2-1-1-4-2-2-1/1-⊗-2⊗8
2	Pant13k 2093	Pop31⊗18-2-1-4-2-2-1/1-⊗-2⊗10A
3	Pant13k 2094	Pop31⊗18-2-1-1-4-2-2-1/1-⊗-1⊗4
4	Pant13k 2096	YHP-B⊗130-2-2-5-1-4-2-1
5	Pant13k 2098	YHP-B⊗161-1-4-1-2-2-1-2-1-⊗-1⊗1
6	Pant13k 2099	YHP-B⊗45-1-2-3-1-6-9-⊗-1⊗5
7	Pant13k 2100	Pob445⊗101-3-2-BBB-⊗-1⊗3A
8	Pant13k 2101	Pob445⊗101-3-2-BBB-⊗-1⊗3A
9	Pant13k 2102	Pob446-74-2-3-B-B-B
10	Pant13k 2103	POB.45 C8-251-1-2⊕1
11	Pant13k 2105	Pob.45 c8-86-1-1-1-8-⊕-1
12	Pant13k 2106	Tarun⊗83-1-3-2
13	Pant13k 2108	DMR Hyd-1702
14	Pant13k 2109	DMR Hyd-1719
15	Pant13k 2110	DMR Hyd-1961
16	Pant13k 2111	DMR Hyd-1284
17	Pant13k 2112	DMR Hyd-1284
18	Pant13k 2113	P1-3
19	Pant13k 2114	HK1 162
20	Pant13k 2115	DTPYC9-F67-2-2-1-3-2-1-3-2-1-3-B-B-B
21	Pant13k 2116	S99TLYQ-HG-AB*4-20-BBB
22	Pant13k 2117	WLS-F69-3-12-1-B-1-B-B
23	Pant13k 2118	WLSC 9010
24	Pant13k 2119	WLSC 9119
25	Pant13k 2120	CIMMYT material
26	Pant13k 2121	CIMMYT material
27	Pant13k 2124	Tr.67/6406
28	Pant13k 2125	Tr.61/7117
29	Pant13k 2126	Tr.61/7117
30	Pant13k 2128	Pant12k/QPM1-2/4061⊕
31	Pant13k 2130	Pant12k/QPM1-2/4071⊕
32	Pant13k 2132	CM 300

between beta-carotene with grain color and observed poor correlations with low R^2 values which indicated that kernel color based visual selection may not be effective in selection of lines for high beta-carotene. Instead, marker assisted selection (MAS) may prove much more

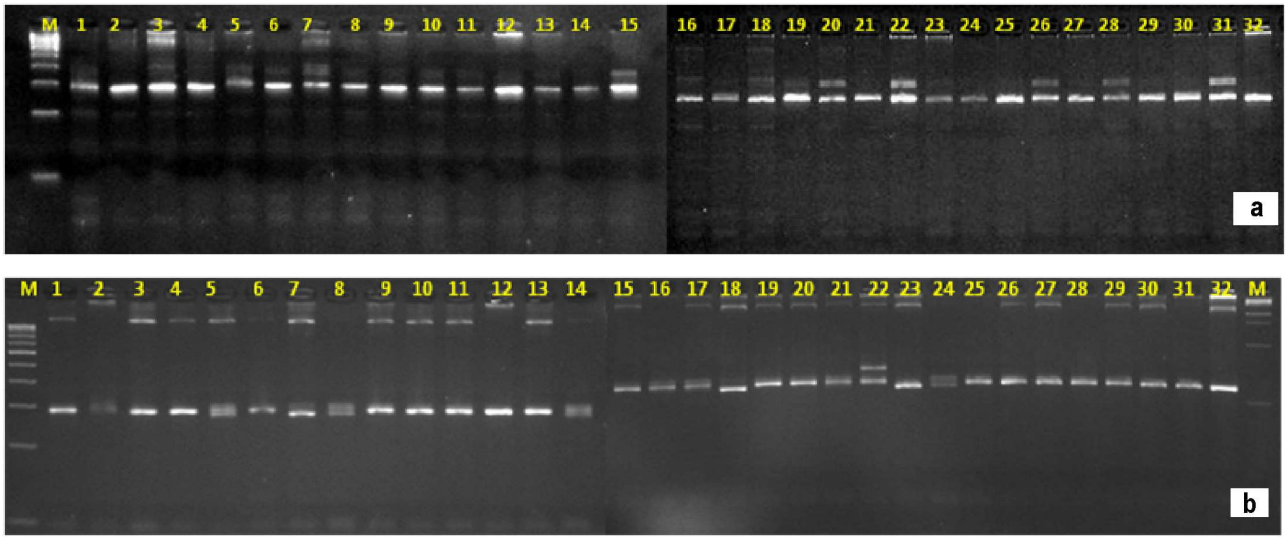

Fig. 4. Genotyping of LcyE and CrtRB1 loci in maize inbred lines; a: Genotyping with marker LcyE 5'TE, b: genotyping with CrtRB1 3'TE

efficient than selection based on color alone.

The experimental material further characterized for *LcyE* and *CrtRB1* genes using gene specific markers (Table 2). Three markers for *LcyE* gene (LcyE 5'TE, LcyE-SNP216, and LcyE 3'TE) and two markers for *CrtRB1* gene (CrtRB15'TE and CrtRB1 3'TE) were used in the study. The favorable alleles of each markers were reported by Hirschberg [8] for *LcyE* and Yan *et al.* [9] for *CrtRB1* markers. When the amplified products analyzed (Fig. 4 a and b), it was found that the amplified alleles in the experimental material were in accordance with the nomenclature provided by them except LcyE-SNP216, CrtRB1 3'TE and CRTRB1 5'TE. Babu *et al.* [21] reported that markers were diagonistic of causative polymorphisms within *LcyE*, developed based on temperate germplasm, and were frequently found not to be reproducible in tropical and sub-tropical genetic backgrounds. While these functional DNA markers can be used to facilitate the development of maize cultivars fortified with high provitamin A, their efficacy in breeding lines has not been fully elucidated. In case of *CrtRB1* 3' TE, we found six amplified alleles including 296+1221 bp in 8 genotypes. The quantitative data on amount of beta-carotene showed that the genotypes used in the experiment has moderate amount of beta-carotene i.e. in range of 3.5 µg/g to 4.814 µg/g. The amplified products of *LcyE* 5'TE and *LcyE* 3'TE were not in accordance with the earlier reports [8], while in 4 genotypes, *LcyE*-SNP216 had amplified 395 bp allele which is favorably associated with low branching flux of β carotene. Among these 4 genotypes, only one showed higher beta-carotene while other 3 had low value for beta-carotene. The 800 bp amplified product by *CrtRB1* 5'TE found in all the genotypes except seven which showed 860 bp alleles.The weaker association between *LcyE*-SNP216 genotypes and beta-carotene in this study is not surprising if we consider that several other genes regulate the flux in carotenoid biosynthesis and that hydroxylation reactions (*CrtRB1*) also act on beta-carotene content. As emphasized by Vallabhaneni *et al.* [22], *LcyE* is effective in controlling the pathway branching, but does not necessarily results in enhanced accumulation of beta-carotene content due to hydroxylation of beta-carotene to other carotenoids. The combined effect of *LcyE* and *CrtRB1* alleles produced higher level of beta-carotene noted in the one of the lines which had favorable alleles for *LcyE*-SNP216 and *CrtRB1* 3'TE. One possible reason for such disparity may be because of complex heritability of carotenoids and environmental effects in upward and downward estimates of the carotenoids. Multilocation evaluation may help in minimzing GxE interaction of kernel carotenoids. Azmach *et al.* [23] reported the similar situations while screening breeding lines used for maize variety delivery to Sub-Saharan Africa. Studies have examined the individual and combine effects of the functional polymorphisms of these two genes where it has been observed that the proposed diagnostic polymorphisms for *LcyE* could not distinguish between inbred lines with high lutein and high zeaxanthin, representing carotenoids in the α and β branches of the pathway [3]. Inbred lines identified with high beta-

carotene identified were consisted of unfavourable alleles for *CrtRB1* polymorphic markers. Similar inconsistencies for the favourable alleles of the *CrtRB1* 3'TE have been observed [19]. The reported inconsistencies in the effects of the diagnostic DNA markers for the proposed favourable alleles of *LcyE* and *CrtRB1* require further investigation of these markers using diverse inbred lines to identify the most robust and effective markers for marker assisted selection.

The applicability of markers depends upon the extent of polymorphism which is indicated by PIC value. The PIC value of the markers used in the study was found to be vary from 0.21 (LcyE-SNP216) to 0.73 (CrtRB1 3'TE) with mean value of 0.45. The UPGMA (unweighted pair group method with arithmetic mean) dendrogram was constructed using Jaccard's similiarity coefficients of SSR markers data generated on genotypes used in the present study. The dendrogram divided 32 inbred lines into four main groups. The first two groups had lines with high beta-carotene content and best allelic variants for the LcyE-SNP216 and CrtRB1 3'TE. However, these groups also consisted of lines with poor in beta-carotene content and with undesirable allelic variants. The 10 lines of group III and IV consisted of lines with low beta-carotene content. This showed that if combined effects of gene based marker data of CrtRB1 and LcyE-SNP216 can be used for accurate selection of desirable lines for enhancing beta carotene.

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