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Identification of potential donor for pro-vitamin A using functional markers in maize (*Zea mays* L)

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Abstract

Vitamin A deficiency (VAD) is a global health problem. Maize naturally accumulates carotenoids including β-carotene, a major source of vitamin A. Thirty-seven Indian maize genotypes were screened for total carotenoids and β-carotene content. A positive significant association of kernel colour was observed with total carotenoid but not for β-carotene. The level of β-carotene among the selected 37 genotypes was low (0.14 to 4.63µg/g) as compared to total carotenoids (15.94 to 66.46 µg/g) thus necessitates the introduction and deployment of lines with high β-carotene. Hence, 54 inbred lines developed under the HarvestPlus programme were screened with functional markers of *lcyE* and *crtRB1* genes of the carotenoid biosynthetic pathway. Three genotypes (HP704-13, HP704-22, and HP704-23) amplified most of the favorable alleles for both these genes. However, due to long Anthesis Silking Interval (ASI) and poor adaptation HP704-13 and HP704-23 could not be maintained. Under normal storage condition genotype, HP704-22 recorded 7.32 µg/g of β-carotene which can be used as a donor for provitamin-A or to diversify the Indian germplasm.

Key word: Maize, carotenoids, β-carotene, *lcyE* & *crtRB1*, bio-fortification

Introduction

Vitamin A deficiency (VAD) is a global health problem affecting 140 to 250 million children (WHO 2009). Vitamin A is not synthesized in the human body which makes us dependent on plant-derived dietary provitamin A. Fruits and leafy vegetables are the major sources of vitamin A, but because of high cost and low purchasing power people from developing nations cannot fulfill their dietary requirements. Therefore, bio-fortification can be the most economic and effective way to eliminate dietary deficiencies for vitamin-A, because maize is an essential staple cereal crop in many countries that naturally accumulate carotenoids in the edible seed endosperm and exhibit wide genetic diversity with the corresponding variation in carotenoid profiles (Harjes et al. 2008). Thus, maize is an obvious target of bio-fortification for provitamin-A.

In the carotenoid biosynthetic pathway, phytoene synthase (PSY) is the first enzyme to condense two molecules of geranyl geranyl pyrophosphate (GGPP) to a single molecule of phytoene (Buckner et al. 1990). Subsequently, the enzyme phytoene desaturase (PDS) converts phytoene to zeta-carotene by a two-step desaturation process. Zeta-carotene desaturase (ZDS) is the third enzyme to convert zeta-carotene to lycopene which is the branching point where lycopene can either go to produce β-carotene or α-carotene depending on the relative activity of enzymes *i.e.* lycopene beta cyclase (LCYB) and lycopene epsilon cyclase (LCYE), respectively. Both β-carotene and α-carotene independently can undergo hydroxylation and produce zeaxanthin and leutin, respectively but these components lack provitamin A activity (Raveendran and Valarmathi, 2020). The hydroxylation intermediate in the beta branch is β-cryptoxanthin, with only one beta-ionone ring similar to α-carotene. Hence α-

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carotene and β-cryptoxanthin have half the provitamin A activity compared to β-carotene. Hydroxylation of the carotene diminishes the provitamin A pool by converting provitamin A compounds to non-provitamin A xanthophylls. To increase the β-carotene (having highest provitamin A activity) level in maize kernel, there is a need to combine two approaches, (i) increasing carotenoid pool toward β-branch rather than α-branch, (ii) stop the downward hydroxylation steps of carotene components which produce xanthophylls. Therefore, pathway branching and hydroxylation are major determining steps to regulate provitamin A levels and the two most important genes involved in this pathway branching and hydroxylation are *lycopene epsilon cyclase* (*lcyE*) and *β-carotene hydroxylase1* (*crtRB1*), respectively. Harjes et al. (2008) and Yan et al. (2010) independently reported favourable alleles for *lcyE* and *crtRB1*, respectively that increases kernel β-carotene content. They also reported PCR-based functional markers for these genes which can be used in marker-assisted selection (MAS). In India, appreciable work has been done to enhance the provitamin A content of maize hybrids through marker-assisted backcross breeding. Vignesh et al. (2014) transferred the *crtRB1* allele into seven elite maize inbreds, *viz.*, VQL1, VQL2, V335, V345, HKI1105, HKI323, and HKI161. Further, Zunjare et al. (2018) stacked the *crtRB1* and *lcyE* alleles in the QPM background. However, the donors' parents carried only one polymorphic site in the *crtRB1* and *lcyE* genes. So, the opportunity to further improve β-carotene exists by combining both the genes carrying more number of favourable polymorphism. CIMMYT, under the HarvestPlus programme has developed several genotypes rich in provitamin A and many of them are being utilized in several MAS programme. The present investigation was carried out to know the variability for total carotenoid and β-carotene in a set of 37 selected Indian lines and to identify the best donor line(s) using functional markers in a set of 54 CIMMYT inbreds which carry the most favorable allele(s) of *crtRB1* and *lcyE* and accumulate higher provitamin A.

Materials and methods

Germplasm

The genotypes characterized include inbreds developed at ICAR-IIMR, accessions imported from CIMMYT, Mexico and indigenous accessions (IC) procured from NBPGR (Table 1). Thirty-seven genotypes for carotenoid and β-carotene estimations were selected

Table 1. A list of genotypes screened for total carotenoid and β-carotene contents

	Genotypes	Origin	Kernel colour	Av. carot-enoid (µg/g)	Av. β-car otene (µg/g)
1.	MRCHY5789-3-1-1-2-2	IIMR	Y	17.62	3.04
2.	MRCHY4856-3-1-3-1-1	IIMR	LO	29.67	4.36
3.	MRCHY5782-2-2-1-2-1	IIMR	P	31.51	4.63
4.	MRCHY5782-2-2-1-2-1	IIMR	LO	30.06	1.41
5.	MRCHY5482-1-1-1-2-1	IIMR	LO	35.37	0.43
6.	MRCHY5782-2-2-1-2-1	IIMR	Y	29.46	0.49
7.	MRCHY5482-3-3-1-2-1	IIMR	DO	39.57	0.65
8.	HEYpool-B-B-52-1	IIMR	LO	22.51	1.27
9.	HEYpool-B-B-50-2	IIMR	DY	16.81	0.87
10.	HEYpool-B-B-43-1	IIMR	Y	16.46	0.83
11.	HEYpool-B-B-55-1	IIMR	LO	33.96	1.66
12.	HEYpool-B-B-60-3	IIMR	Y	25.17	0.90
13.	HEYpool-B-B-50-1	IIMR	DY	26.54	2.54
14.	HEYpool-B-B-50-1	IIMR	DO	43.00	2.24
15.	HEYpool-B-B-55-2	IIMR	LO	41.33	3.80
16.	HEYpool-B-B-60-2	IIMR	Y	24.70	3.40
17.	HEYpool-B-B-41-3	IIMR	Y	17.75	1.48
18.	HEYpool-B-B-51-1	IIMR	Y	34.17	1.20
19.	HEYpool-B-B-48-6	IIMR	LO	36.36	1.16
20.	HEYpool-B-B-53-2	IIMR	LO	36.92	1.36
21.	IC-655835-B-⊗-⊗	NBPGR	LO	34.17	1.22
22.	HEYpool-B-B-61-3	IIMR	Y	25.00	1.08
23.	HEYpool-B-B-48-4	IIMR	Y	30.40	0.87
24.	HEYpool-B-B-56-1	IIMR	LO	25.94	0.94
25.	IC-639247-B-⊗-⊗	NBPGR	Y	51.92	1.37
26.	IC-639392-B-⊗-⊗	NBPGR	DO	37.47	0.96
27.	HEYpool-B-B-34-2	IIMR	Y	24.14	0.31
28.	HEYpool-B-B-47-2	IIMR	DY	15.95	0.14
29.	HEYpool-B-B-44-9	IIMR	Y	19.34	1.73
30.	IC-639563-B-⊗-⊗	NBPGR	Y	52.44	1.00
31.	IC-568299-B-⊗-⊗	NBPGR	DO	45.62	0.80
32.	IC-639564-B-⊗-⊗	NBPGR	Y	25.77	0.15
33.	IC-632050-B-⊗-⊗	NBPGR	L O	66.46	0.28
34.	IC-639486-B-⊗-⊗	NBPGR	Y	30.53	0.41
35.	IC-639428-B-⊗-⊗	NBPGR	LO	49.22	1.28
36.	IC-639253-B-⊗-⊗	NBPGR	L O	51.66	1.31
37.	IC-639337-B-⊗-⊗	NBPGR	LO	29.16	0.51

Av. = Average, (Y = Yellow, DY = Dark yellow, LO = Light orange, DO = Dark orange and P = Purple)

based on the variation in kernel colour, whereas 54 inbreds which were procured from CIMMYT for validation of functional markers linked to *crtRB1* and *lcyE* were developed at CIMMYT to serve as provitamin A donors with varying level of provitamin A and adaptation.

Isolation of genomic DNA and PCR reaction

Genomic DNA was isolated from leaf samples of three-week-old seedlings using the standard CTAB method (Saghai-Maroof et al. 1984). Reported primer pairs by Harjes et al. (2008) and Yan et al. (2010) for two genes (*lcyE* and *crtRB1*) of the carotenoid pathway were used. PCR amplification was carried out with standard PCR protocol (35 cycles of denaturation at 94p C for 45s, annealing at 60p C for 45s, and extension at 72p C for 45s) with an initial denaturation of 94p C for 4 min.

Carotenoid extraction, estimation

For carotenoid extraction selfed ear of each genotype was harvested separately and carefully dried up under shade to avoid direct sunlight. After proper drying, the harvested ears were kept in a cool chamber, kernels from the middle of the ear were taken out and ground. The flour after grinding was stored at –20°C until extraction is done. Carotenoid extraction protocol, developed by Torbert Rochefords' Lab, for the extraction of carotenoids from matured maize kernels was used (Schaub et al. 2004). All the laboratory activities were done in dim light conditions to avoid degradation of carotenoids. Estimation of total carotenoids and β-carotene was carried out through column chromatography. The analysis of variance of carotenoid content and β-carotene was carried out based on two replication and in complete randomized design (CRD) using SPSS (Version 16.0). Mean values for total carotenoid and β-carotene were used to determine superior genotypes.

Functional markers for crtRB1 and lcyE gene

A set of primers developed by Yan et al. (2010) for three polymorphic sites namely, 52 TE (crtRB1 H1UF and crtRB1 H1UR), InDel4 (crtRB1D4F and crtRB1 D4R), and 32 TE (crtRB1 65F, crtRB1 62R, and crtRB1 66R) identified in *crtRB1* gene of maize through association study were used. Three distinct amplicon size at 52 TE *viz.*, 800 bp (allele 1: with 397 bp insertion), 600 bp (allele 2: with 206 bp insertion) and 400 bp (allele 3: without insertion) determines varying levels of β-carotene and the primers used to amplify were H1UF and H1UR. Among three amplicons, allele 2 contributes most toward β-carotene accumulation followed by allele 3 and allele 1, respectively. Similarly, at 32 TE, three primers (65F, 62R, and 66R), flanking the insertion (65F and 66R) as well as located within the insertion element (62R), were used together to amplify three distinct allelic combinations. One amplified fragment is possible when no insertion (allele 1: 543 bp), whereas allele 2 and allele3 has more than one amplified fragments because of insertion with 296 bp in common. The allelic hierarchy to β-carotene concentration is allele1 > allele3> allele2. InDel4 polymorphic site had 12bp insertion/deletion with two alleles of size 129 bp (allele 1) and 117 bp (allele 2) and allele 1 contribute more for β-carotene concentration.

Three key polymorphic sites (52 InDel in the promoter, one SNP at position 216, and 32 InDel in UTR) in *lcyE* gene significantly affect β-carotene concentration in maize endosperm (Harjes et al. 2008). At 52 end, there was insertion at 2 points and four primers namely TE103PF F-1, TE103PR R-1, ZGt111204-976R(1) F1, and TE105PR R1 in different combination were used to amplify four different possible classes of amplicons *i.e.* class 1 (150 + 280 bp), class 2 (250 bp), class 3 (250 + 380 bp) and class 4 (993 bp). Class 2 and class 4 are two different allelic fragments of the same primer pair ZGt111204-976R(1) F1 and TE105PR R1, class 4 is a larger fragment because of insertion. In addition to class 2 and class 4, multiplex PCR involving 3 primer set could result class 1 (ZGt111204-976R(1) F1 + TE103PR R-1 and TE103PF F-1 + TE103PR R-1) and class 3 (ZGt111204-976R(1) F1 + TE105PR R1 and TE103PF F-1 + TE105PR R1). For 32 TE, two classes are expected, D (144 + 502 bp) and I (399 + 502 bp) [I and D acronyms as mentioned by Harjes et al. (2008)]. 144 bp and 399 bp size fragments are amplified by deletion (3pINDL-L2 + 3pINDL-R2) and insertion (3pINDL-L1 + 3pINDL-R1) specific primers respectively whereas 502 bp (3pINDL-L2 + 3pINDL-R1) is wild type allele. Allelic combination of class D increases β-carotene and zeaxanthin branch of the carotenoid biosynthetic pathway.

Results

Variation in kernel carotenoids and β-carotene

Analysis of variance (ANOVA) in a completely randomized design (CRD) revealed significant variation between the genotypes for total carotenoid as well as

for β-carotene. The total carotenoid levels in IC-639247-B-⊗-⊗, IC-639563-B-⊗-⊗, IC-632050-B-⊗-⊗ and IC-639253-B-⊗-⊗ was far superior (>50 μg/g), whereas the beta-carotene content in the following genotypes namely, MRCHY5789-3-1-1-2-2, MRCHY 4856-3-1-3-1-1, MRCHY 5782-2-2-1-2-1, HEY pool-55-2 and HEY pool-60-2 was > 3μg/g. However, the range of variation for kernel carotenoid was as low as 15.94 μg/g to as high as 66.46 μg/g with a mean of 32.54 μg/g (Table 1). The results indicated considerable variation for total carotenoid but the level of β-carotene content is very low against the set target of 15 μg/g by the HarvestPlus program to meet the Recommended Daily Allowance (RDA).

Correlation between kernel colour, β-carotene, and total carotenoid

The genotypes were also classified into different groups based on their kernel colour. Fifteen and three genotypes had yellow (Y) and dark yellow (DY) kernel, respectively whereas 14 and four genotypes had light orange (LO) and dark orange (DO) kernel, respectively. Only one genotype had purple kernel (P) colour. In the Y group, β-carotene ranged from 0.15 μg/g to 3.4 μg/g and total carotenoid ranged from 16.46 μg/g to 52.44 μg/g whereas the range for β-carotene and total carotenoid in the DY group was 0.14 μg/g to 2.54 μg/g and 15.95 μg/g to 26.54 μg/g, respectively. Level of β-carotene and total carotenoid varied from 0.28 μg/g to 4.36 μg/g and 22.51 μg/g to 66.46 μg/g, respectively in the LO group and 0.65 μg/g to 2.24 μg/g and 37.47 μg/g to 45.62 μg/g, respectively in DO group. Mean β-carotene was 1.22 μg/g, 1.18 μg/g, 1.50 μg/g, 1.16 μg/g, and 4.63 μg/g in Y, DY, LO, DO, and P group, respectively (Fig. 1). Similarly, mean total carotenoid in Y, DY, LO, DO and P group was 28.32 μg/g, 19.77 μg/g, 37.34 μg/g, 41.42 μg/g, and 31.51 μg/g, respectively. Multiple correlations between kernel colour, total carotenoid, and β-carotene were carried out to investigate any association of total carotenoid and β-carotene with kernel colour. A positive significant correlation was observed between kernel colour and total carotenoid (0.393; p-value 0.000) whereas β-carotene did not results in any association either with kernel colour or with total carotenoid.

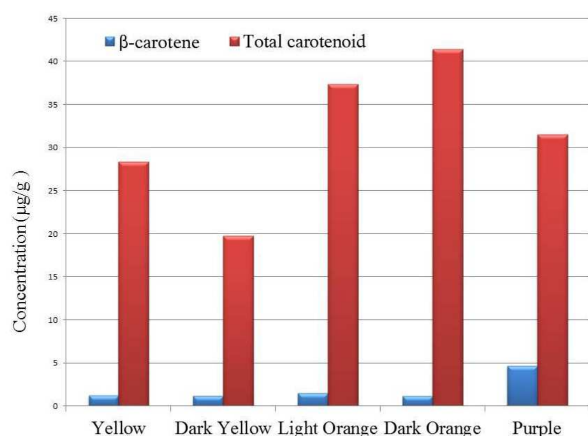

Fig. 1. Mean β-carotene and total carotenoid according to kernel colour

The 54 CIMMYT inbreds were screened with primers of the two reported genes (*crtRB1* and *lcyE*) to identify the best donor line(s). PCR assay for *crtRB1* was carried out for all the three functional polymorphisms using four pairs of markers, *viz.,* H1UF + H1UR (52 TE), D4F + D4R (InDel4), 65F + 62R, and 65F + 66R (32 TE). Three lines, *viz.,* HP704-13, HP704-22, and HP704-23 were found to amplify the most favorable allele of 32 TE (543 bp) along with 296 bp allele and 52 TE (600 bp). Whereas for the InDel4 polymorphic site, the alleles of the expected class (129 bp and 127 bp) did not amplify in this study, instead a different band (approximately 350 bp) was amplified in most of the genotypes. Hence, genotypes HP704-13, HP704-22, and HP704-23 amplified the most favourable haplotypic combination for the *crtRB1* gene (Fig. 2).

Fig. 2. Characterization of genotypes using primers of *crtRB1*, Lanes:1=HP704-9, 2=HP704-10, 3=HP704 -13, 4=HP704-14, 5=HP704-22 and 6=HP704-23

Screening with primers for *lcyE* gene resulted in amplification of 250 bp (class 2) fragments in all the genotypes, however, the favorable allele, class 4 did not amplify. Similarly, for the class 3 fragment, TE103PF F-1 + TE105PR R1 (R2) primer pairs did not result in any amplification. Though an unexpected allele of 100 bp was observed instead of 150 bp for class 1 fragment, and no amplification was observed for the 280 bp allele. For 32 TE, most of the genotypes recorded positive bands of favorable class D in different uniplex PCR reactions. Twenty-nine and 33 genotypes amplified 144 bp and 502 bp fragments, respectively. All the 29 genotypes amplified 144 bp

fragment also showed the presence of 502 bp allele (Fig. 3).

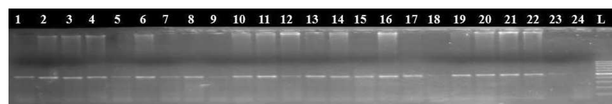

Fig. 3. Characterization of genotypes by primer L2 and R1 (lcyE-3'E) . Lanes: 1=HP704-9, 2=HP704-10, 3=HP704 -13, 4=HP704-14, 5=HP704-22, 6=HP704-23,7=HP963-13, 8=HP963-14, 9=HP963-15, 10=HP963-17, 11=HP963-20, 12=HP963-21, 13=HP963-26, 14=HP963-27, 15=HP963-32, 16=HP963-33, 17=HP963-34, 18=HP963-35, 19=HP963-37, 20=HP963-42, 21=HP963-43, 22=HP963-44, 23=HP963-45, 24=HP963-47, 25=HP963-49, 26=HP963-51, 27=HP963-52, 28=HP963-1, 29=HP917-2, 30=HP963-4, 31=HP963-6 32=HP963-9 and 33=HP963-1,) Lanes 25-33 not shown in figure

Kernel carotenoids and β-carotene of most favourable genotypes

Three genotype HP704-13, HP704-22, and HP704-23 showed the most favourable haplotypic combination for both *crtRB1* and *lcyE* however, due to long Anthesis Silking Interval (ASI) and poor field performance genotypes HP704-13 and HP704-23 could not be maintained. Thus, kernel carotenoids and β-carotene of only HP704-22 was estimated under normal storage condition which was 7.32 µg/g and 26.80 µg/g, respectively.

Discussion

The variation of total carotenoids observed in the present investigation is comparable to those reported earlier. A wide range of total carotenoid contents *viz.,* 9.90-39.96 µg/g (Hulsof et al. 2007), 5.5-66 µg/g (Harjes et al. 2008), 0.94-38.25 µg/g (Das et al. 2012) have been reported earlier. However, variation for β-carotene content was very less (0.14-4.63 µg/g) with a mean of 1.40 µg/g which conforms with previous studies on maize reported by Egessel et al. (2003) (0.5 to 3.4 µg/g) and Menkir et al. (2008) (0.7 to 4.7 µg/g). Although different investigations reported a diverse range for kernel β-carotene, most of the yellow maize consumed around the world is having <2 µg/g of provitamin-A (Pixley et al. 2013). A positive correlation was observed between kernel colour and total carotenoid. Harjes et al. (2008) also observed poor correlations of β-carotene and total carotenoids with grain colour with low R^2 values. Hence, indirect selection based on kernel colour may be partly effective to improve total carotenoid but not for β-carotene.

Lack of required amount of variation for β-carotene and lack of association with morphological marker necessitates the use of exotic germplasm as a source of provitamin-A which can be used as a donor for marker-assisted gene introgression or to diversify the adapted germplasms under use. Maize genotypes with more number of the positive allele of *crtRB1* and *lcyE* will ensure a higher concentration of provitamin-A in the kernel. Further, a single donor parent carrying desirable alleles of both the genes permits the transfer of both the genes in a single step which avoid stepwise or simultaneous transfer where a separate background selection programme for a recurrent parent becomes necessary. Screening and selection of best genotype carrying positive alleles for these genes based on PCR assay is an alternative to the phenotypic selection of β-carotene-rich maize genotypes using chromatography, which is a time-consuming and expensive affair. The most favorable haplotypic combination for the *crtRB1* gene is allele 2 of 52 TE (600 bp) and allele 1 of 32 TE (allele 1) irrespective of any allele at InDel4 (Yan et al. 2010). In the present study, three genotypes, namely, HP704-13, HP704-22, and HP704-23 were found to carry favorable alleles at 52 TE and 32 TE. The highest β-carotene content was reported when the favourable alleles of 52 TE (allele 2) and 32 TE (allele 1) were combined irrespective of any allele at InDel4 (Yan et al. 2010). Therefore, though at InDel4, none of these genotypes have promising allele, then also these can be utilized as donors for the *crtRB1* gene.

In comparison to *crtRB1*, large number of genotypes were carrying the most favorable classes at 32 TE of the *lcyE* gene. As mentioned above, markers for *lcyE* 52 TE amplified a different band rather than expected which could be either due to non-specific PCR amplification or the presence of a different allele. Similar results had been reported by Rashmi et al. (2014) where novel alleles other than reported earlier for *lcyE* 52 TE and *lcyE* 32 TE were observed. So, it is convenient to use 32 TE primers to identify positive alleles for *lcyE* in this investigated germplasm. Though *crtRB1* 52 TE had amplified best classes in few genotypes we have found that the amplification is not consistent even with the same reaction condition. These genes may reside in PCR-recalcitrant genomic regions and may require different assay procedures for effective screening. Yan et al. (2010) reported that

crtRB1 and *lcyE* have a joint additive effect on β-carotene concentrations in maize endosperm. Zunjare et al. (2018) stacked the favourable alleles of *crtRB1* (32 TE) and *lcyE* (52 TE) in the four QPM hybrids, *viz*., HQPM1, HQPM4, HQPM5, and HQPM7 which resulted in a 4.5-fold increase in provitamin A (9.25-12.88 μg/g). So, genotype HP704-22 which recorded the most favorable classes for both the genes can be used as the best donor for marker-assisted introgression in suitable recipient parents of selected hybrids.

Authors' contribution

Conceptualization of research (AKD, SR); Designing of the experiments (AKD, CGK); Contribution of experimental materials (AS, RK, GM, VS); Execution of field/lab experiments and data collection (DPC, Sapna, YKR); Analysis of data and interpretation (AKD, SR, CGK); Preparation of the manuscript (AKD, CGK, BK).

Declaration

The authors declare no conflict of interest.

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