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Screening of *Zea mays* L. Germplasm for the High Carotenoid Endosperm using Biochemical and Molecular Approaches

G. Adinarayana[1], V. Ratnakar[2], D. Ramya[2], S. Vijay[2], G. Pavan Kumar[2], P. Sateesh Kumar[2], K.R.S. Sambasiva Rao[1,3]

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ABSTRACT

Background: In the rapid growing and busy world, the hidden hunger for the micronutrients, vitamins and protein is increasing on a high rate. Among which retinol is the key vitamin which cannot be synthesized by the humans and need to be supplied in the form of carotenoids.
Methods: The present study focused in screening the best maize line with high carotenoid content. Carotenoid content was tested and quantified using HPLC. ε-LCY gene and β-HYD 3 were used to screen the best maize line.
Result: Among 8 maize lines tested, 5732 showed high carotenoid content *i.e.*, 24.47 µg/g when tested using conventional UV spectrophotometer studies. Quantification of individual carotenoid component was done by HPLC which showed lutein 7.548 µg/g, zeaxanthin 3.21 µg/g, β-cryptoxanthin 5.691 µg/g and β- carotene 8.455 µg/g were recorded in 5732 maize cultivars. Molecular screening using ε-LCY gene and β-HYD 3 revealed 5732 as the best carotenoid maize line with high carotenoid favorable alleles which can be further used for the inbreeding programs.

Key words: Carotenoid, Maize, β-carotene, β-HYD 3, ε-LCY gene.

INTRODUCTION

Retinol is a key essential vitamin required by the humans which cannot be synthesized and needed to be given as a food supplement as provitamin A carotenoids which include β-carotene, α-carotene and β-cryptoxanthin which are the key precursors for the retinol biosynthesis. One of the best methods to improve the retinol content in the food is to breed the plant varieties for high β-carotene (Vignesh *et al*., 2015). In this scenario, the best suitable crops for the enrichment of β-carotene are maize.

Zea mays L. is the third largest cultivated crop in the world after wheat and paddy and is termed as "Queen of all the cereals" (Sukumar *et al.,* 2023). It is a staple meal for millions of people and it is grown in an area of 168 million hectares with a total output of 984 million tons with an average output of 3.41 tons/ha. In India, maize is cultivated across 9.4 million hectares in 2013-14 with a total yield of 16.8 million metric tons and an average output is 2.5 tons/ha. The major compounds in the maize grains are β- carotene, β-cryptoxanthin, zeaxanthin and lutein. Among which β-carotene has the major provitamin A content and is present in very low concentrations in maize grains. So, screening of germplasm for high β-carotene maize inbred varieties and transferring the high β- carotene alleles from superior lines to the adapted cultivars through introgr
ession breeding are much encouraged (Harjes *et al.,* 2008).

Maize kernels have different colors like white, yellow and orange which depends on the presence or absence of carotenoids. Yellow and orange varieties of maize contain carotenoids in its endosperm whereas the white variety lacks the carotenoid. Recent studies have also led to the elucidation of the carotenoid biosynthetic pathway in plants and identification of candidate genes and favorable alleles that influence the synthesis of carotenoids (Babu *et al.,* 2013). Among several genes influencing the carotenoid biosynthesis, three genes *viz*., *Phytoene syntheses 1*(*Y1 or Psy1*), *lycopene epsilon cyclase* (*lcyE*) and β-*carotene hydroxylase 1* (*crtRB1*) play a crucial role in the final accumulation of carotenoids in the endosperm (Yan *et al.,* 2010).

The present study aims in studying the β-carotene content in the maize lines and identification of β-carotene alleles.

[1]Department of Biotechnology, Acharya Nagarjuna University, Guntur-522 510, Andhra Pradesh, India.
[2]Department of Biotechnology, Nuziveedu Seeds Limited, Hyderabad-501 401, Telangana, India.
[3]Department of Pharmacy, Mangalayatan University, Jabalpur-483 001, Madhya Pradesh, India.

Corresponding Author: K.R.S. Sambasiva Rao, Department of Pharmacy, Mangalayatan University, Jabalpur-483 001, Madhya Pradesh, India. Email: krssrao@yahoo.com

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MATERIALS AND METHODS

Experiment conducted

The experiment was conducted in the year 2021-August, in the R and D laboratory of Nuzuveedu Seeds Private Limited, Hyderabad.

Extraction of total carotenoids

Total carotenoid was estimated from the individual genotypes using the standard protocol (Menkir *et al.,* 2008). After kernel maturation, ears with the husk were manually harvested and were dried under the shade till 14% moisture was obtained. 50 kernels from each genotype were randomly sampled. Sample was ground and 0.5 g of each triplicate per sample was collected. Each step prior to and during biochemical analysis was carried out under the dim yellow light to prevent the photo-oxidation of carotenoid.

0.5 g of fine powder was taken and 6 ml of EtOH:BHT was added to each sample and mixed by vortex mixer. Samples were incubated at 85°C in water bath for 5 min and were mixed again after 3 min by vortex. Freshly prepared 120µL KOH (1g KOH/ml of water) was added to each sample. Saponification was done by incubating samples for 5 min at 85°C in water bath, vortexed for 10 sec and further incubated at 85°C for 5 min. Samples were cooled down on ice packs and 4 mL of water was added. Further 3 mL of petroleum ether (PE):diethyl ether (DE) (2:1 v/v) was added to each sample. Samples were mixed by vortex and thereafter centrifuged at 3600 rpm for 10 min. Upper phase (supernatant) of the mixture was pipetted out and transferred to fresh 50 ml centrifuge tube. Mixing of 3 ml of PE:DE (2:1 v/v) and centrifugation steps were repeated thrice. Approximately, 8 ml of extract was recovered in the final step to which equal volume of PE:DE (2:1 v/v) was added to each sample and mixed by inverting the tube. The absorbance of total carotenoid was measured at 450 nm using UV Vis Spectrophotometer (Elico SL 218) by taking PE:DE (2:1, v/v) as blank*and* the total carotenoid concentration was calculated based on the formulae as suggested by Harvest plus equation (Rodriguez-Amaya and Kimura, 2004).

Sample preparation for the estimation of individual carotenoids

The seeds were cleaned and shade dried. The grains from ears of same families were equally shelled and bulked together*and* were stored in deep freezer (-20°C) for further biochemical analysis. Extraction and quantification of β-carotene (BC), β-cryptoxanthin (BCX), lutein (LUT)*and* zeaxanthin (ZEA), from kernels was done by utilizing standard procedure (Vignesh *et al*., 2015; Kurilich and Juvik, 1999). Under dark conditions, samples of maize seeds were powdered and used for further analysis. Carotenoids from maize samples were extracted by taking 1 g of powder to which 6 mL of ethanol with 0.1% β - hydroxytoulene (BHT) (w/v) was added*and* kept in water bath at 85°C for 5 min with intermittent vortex. To the above hot mixture, 120 µL of potassium hydroxide (KOH) was mixed to saponify the potentially interfering oils. Samples were vortexed and kept in water bath (85°C) for 10 min with intermittent vortex.
After saponification process, samples were kept in the ultra-low freezer and 3 mL of chilled deionized water was added to the samples. 3 mL of petroleum ether (PE): diethyl ether (DE) (2:1 v/v) was added for carotenoids extraction. Samples were centrifuged at 2700 rpm for 10 min. Supernatant was transferred to the new 50 mL of centrifuge tubes and the above steps were repeated thrice for each sample. The organic layers were dried in rotary vacuum evaporator it was dissolved in the tert-butyl-methyl ether (Vignesh *et al*., 2015).

Quantitative analysis of BC and BCX using HPLC

BC and BCX were quantified using Waters and Alliance W2690/5 model HPLC system incorporated with Waters Empoer 3 software with W2998 photodiode array detector. The column used was YMC C30 column (5 µm, 4.6×250 mm). The standards of lutein, zeaxanthin, β-carotene, β-cryptoxanthin were sourced from Sigma Aldrich, Mumbai (Zunjare *et al.,* 2017). The mobile phase used was methanol: TBME (tert-butyl methyl ether) (80:20 v/v)*and* 1 mL/min was the flow rate. Standards were purchased from Sigma Aldrich, India and dissolved in acetone and desired dilutions were made. The concentrations of BC and BCX in each sample was detected by standard regression. To improve the detection of BC and BCX, the absorbance was recorded at 450 nm and the chromatogram obtained was compared with the standards. The pro-A concentration was estimated as sum of BC and half the BCX concentration while non-pro-A estimated as sum of LUT and ZEA concentration (Babu *et al.,* 2013).

Screening of germplasm for the of â–carotene favourable alleles

Maize germplasm from the Nuziveedu seeds Limited, Hyderabad was screened for the β– carotene donors which delivers the superior/favorable genes. Similarly, recurrent parents of high zeaxanthin were also screened and identified. 8 lines of high lutein, high zeaxanthin, β-cryptoxanthin and β -carotene were analyzed. Lutein and zeaxanthin are the 2 compounds which add up to the total carotenoid content as they are abundant in endosperm. Accumulation of β-cryptoxanthin and β -carotene is rare, these compounds get converted to zeaxanthin*and* downstream products towards ABA.

Identifying maize lines with accumulation of â-carotene is a critical factor, with corresponding dominant alleles as they would serve as donors, introgressing superior carotenoid alleles, for controlling the accumulation of β-carotene into high yielding maize elite germplasm lines. As orange color is highly preferred by farmers, most of the germplasm selected were of orange color, *i.e.,* high in zeaxanthin and only very few are yellow in color.

Among those yellow lines, few lines are of enhanced β -carotene, but they are not the best parents in the hybrid crossing program. So, in an obvious sense, the enhanced β - carotene lines were used as donors in crossing program. The orange germplasm high in zeaxanthin was selected as recurrent parents*and* if they are introgressed with β-carotene accumulating alleles, the lines should accumulate β-carotene.

Molecular markers for foreground marker-assisted selection of β -carotene accumulating alleles from donors

PCR assays on both the donors and recurrent parents were performed for target polymorphisms, such as 5′TE, 3′TE and DEL4 present on the genes ε-LCY and β-HYD respectively. PCR products thus amplified were loaded on agarose gels. ε-LCY had 2 polymorphisms such as 5′TE and 3′TE. There were two classes of 5′TE (2 and 3), of which class 2 was favorable (Harjes *et al.,* 2008). An insertion of 8 bp brings a polymorphism of LCY 3′TE. Both the polymorphisms favor an increase in the ratio of zeaxanthin over lutein. However, in recent studies, it was indicated LCYE is not as effective as the β-carotene hydroxylase and it was very obvious, because it had no direct effect on β –carotene accumulation, whereas β-HYD is directly involved in controlling the flux between the conversion of β –Carotene and zeaxanthin. In β-HYD, there were 3 polymorphisms, with 5′TE being most effective (Yan *et al.,* 2010). 5′TE holds two classes, of which class 2 was favorable. Among 3′TE type class 1 with 543 bp was favorable, followed by DEL4 with 12 bp insertion. These markers were used to perform marker assisted foreground selection.

RESULTS AND DISCUSSION

Extraction of total carotenoids

The total carotenoids from the maize cultivars were assessed. A total of 8 maize lines were assessed for total carotenoids content. Among all the cultivars, 5732 cultivar showed 24.47 µg/g followed by 3003 cultivar (20.41 µg/g), whereas low carotenoid content was recorded in 3896 cultivar (7.72 µg/g) Fig 1.

Quantitative analysis of BC and BCX using HPLC

The in-house maize lines were tested for the presence of BC and BCX using HPLC and the components were quantified. Along with the BC and BCX, zeaxanthin and lutein were also quantified. Among all the maize lines tested, high lutein and zeaxanthin were recorded in 5732 *i.e.,* 7.548 µg/g and 3.21 µg/g . Similarly, high β-cryptoxanthin and β-carotene were recorded in 5732 with 5.691 µg/g and 8.455 µg/g Fig 2. The chromatogram of 5732 showed the peak for β- carotene and had secreted high β- carotene content when compared to the maize lines Fig 3.

Most of the maize lines showed very less β- carotene content when compared to the 5732-maize line. When total carotenoid content was estimated, 5732 showed high total

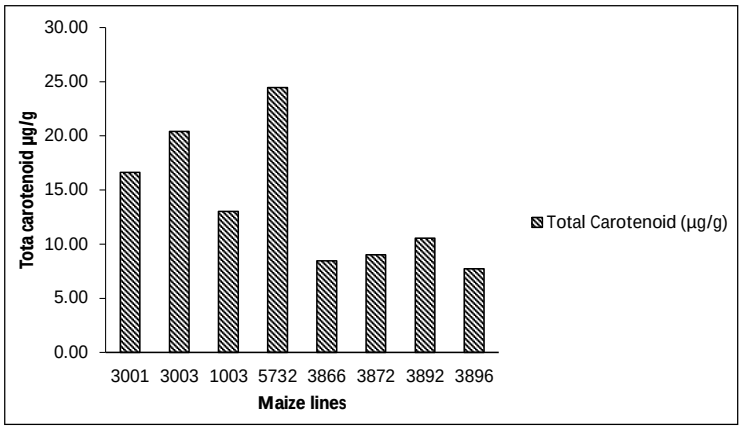

Fig 1: Total carotenoid contents in-house maize lines.

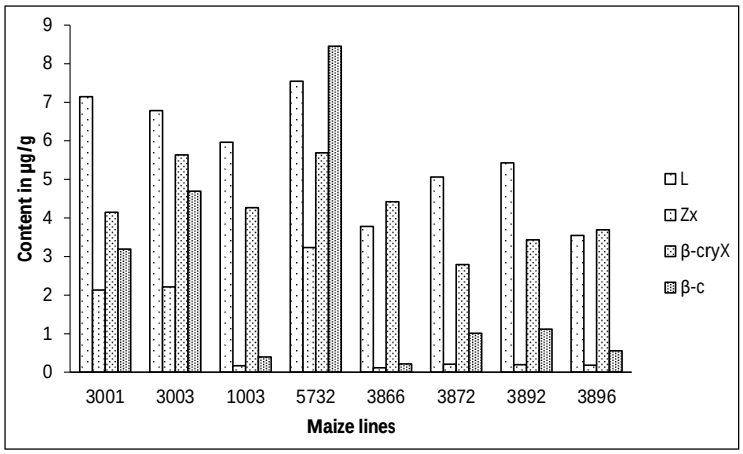

Fig 2: The content of lutein, zeaxanthin, â-cryptoxanthin and â-carotene estimated using HPLC

carotenoid content with 24.925 µg/g followed by 3003 with 19.325 µg/g Fig 4. The results of total carotenoid content obtained in the extraction process matches with the HPLC results. β- carotene is the key component which acts as a precursor for provitamin A. The HPLC analysis shows that the 5732 cultivar has high content of β- carotene and β-cryptoxanthin which are the core precursors in the retinol biosynthesis. Comparatively, the 5732-cultivar showed high synthesis of β-carotene and β-cryptoxanthin when compared to the other cultivars.

Screening of germplasm for the of β–carotene favourable alleles

Screening of germplasm was carried out for the high β–carotene maize lines which has β–carotene favorable genes. Screening process was carried out using SSR markers and percentage of polymorphism observed. Among all the chromosomes (CHR) tested, CHR-5 showed high percentage of polymorphism with 62% when tested using 71 markers, overall 670 markers we tested entire chromosome-1 (Chr) to chromosome-10, we observed average of 44% polymorphism between elite line (3001) and carotenoid donor line (5732), Table 1.

Molecular markers for foreground marker-assisted selection of β-carotene accumulating alleles from donors

Marker-assisted selection of β-carotene accumulating alleles from donors was carried using ε-LCY gene and β-HYD 3. Molecular markers used for the introgressing were ε-LCY gene along with β-HYD 3. The test showed high favorable alleles in class D *i.e.,* 1 followed by class B (8 favorable alleles) (Table 2 and Fig 5).

Carotenoids play a key role in the metabolic activities of humans, further improving the growth and development in the humans. For the Vit A biosynthesis, β-carotene, β-carotene and β-cryptoxanthin act as precursor molecules. Lutein and zeaxanthin act as free radical scavengers which can be taken as an antioxidant dietary supplement to reduce the risk of degenerative diseases (Vignesh *et al.,* 2015). In order to increase the yield of β-carotene in the maize cultivars the inbreeding techniques are the best suitable

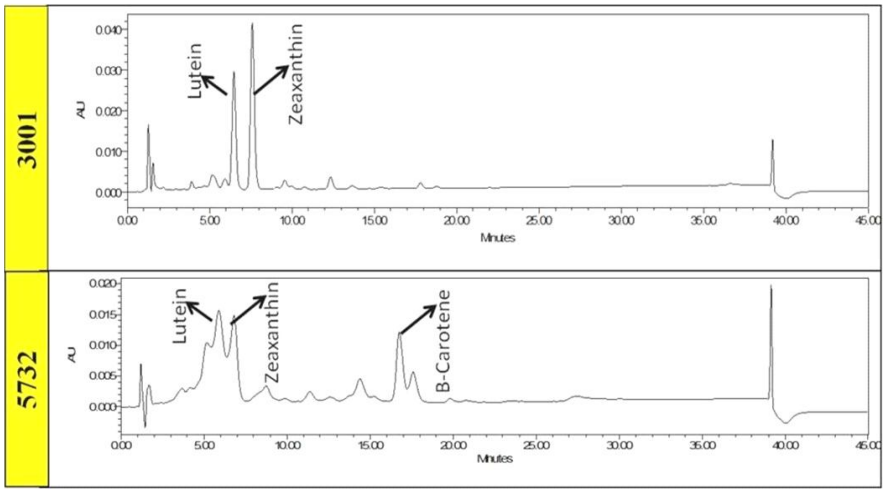

Fig 3: HPLC chromatogram of 3001 and 5732 showing the peak for Lutein, Zeaxanthin and â- carotene.

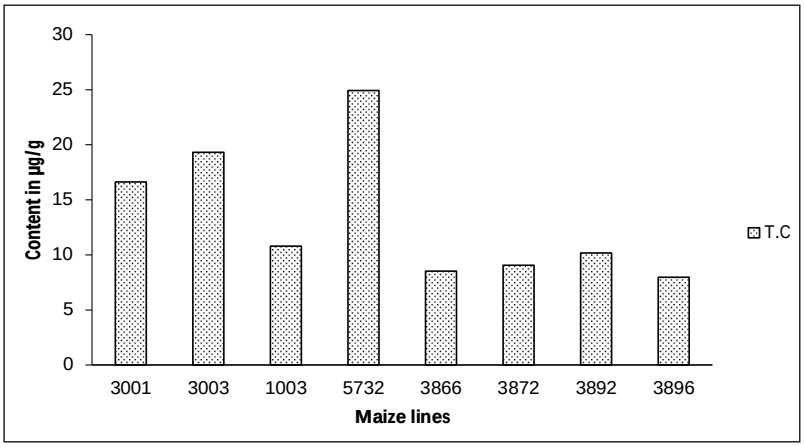

Fig 4: Graph showing the total carotenoid content of the maize lines.

methods. In the present study, total 8 maize lines were selected and the total carotenoid content was tested using the conventional method and also by using HPLC. Among all the maize lines, 5732 cultivar showed 24.47 μg/g when compared to the other maize cultivars. The obtained results are in correlation with the previous results obtained in the Hungarian maize lines which has the total kernel carotenoid in between 12.20-30.10 μg/g (Anshuman *et al.,* 2012). The low total carotenoid in the maize lines show that the cultivars ack the carotenoid favorable alleles. Similarly, the results quantified using the HPLC showed lutein 7.548 μg/g, zeaxanthin 3.21 μg/g, β-cryptoxanthin 5.691 μg/g and β-carotene 8.455 μg/g in 5732 maize cultivar. Among all the 8 maize lines studied for the total carotenoid content, 5732 was selected as high carotenoid containing maize cultivar which indirectly reciprocates the presence of β-carotene favorable alleles. Similar results were obtained in the previous research studies which showed the zeaxanthin 10.22 μg/g, β- cryptoxanthin 1.93 μg/g and β- carotene 2.12 μg/g. The average range of β-carotene is 0-4.58 μg/g (Changan *et al.,* 2017). Similarly, yellow maize lines showed total carotenoid 14 μg/g and 1.69 mg/g for β-carotene (Tura *et al.,* 2012). In the present study the 5732 maize line showed 5.455 μg/g of β- carotene which was much higher than the required level. There are few research reports outlining the genetics of carotenoids in yellow maize, despite the fact that natural genetic diversity in carotenoids has been discovered in yellow maize lines and hybrid types in the temperate zone and in the tropics. Few research reports also indicated the inheritance of gene action in maize (Alhassan *et al.,* 2016; Karuganti *et al.,* 2013).

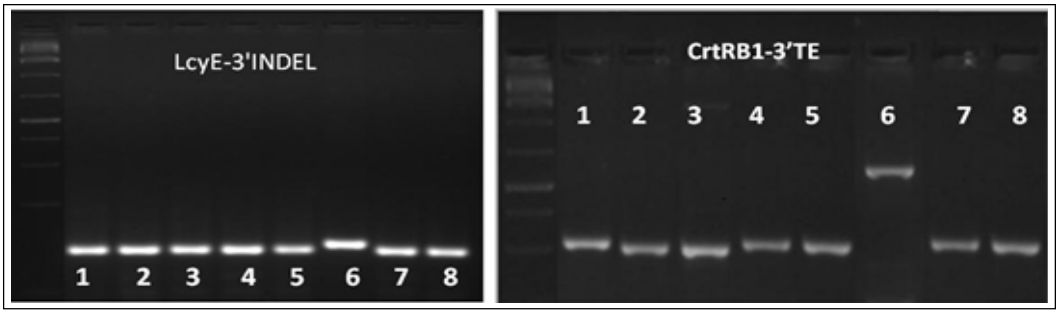

Fig 5: Molecular markers for marker assisted selection of for carotenoid superior alleles for foreground selection å-LCY gene B. â-HYD 3 or CrtRB-1 gene.

Table 1: Number of screened SSRs and percentage polymorphism observed.

Chr. No.	No. of Markers Screened	No. of Polymorphic Markers	% Of Polymorphism
CHR-1	116	39	34
CHR-2	84	35	42
CHR-3	54	22	41
CHR-4	55	29	53
CHR-5	71	44	62
CHR-6	55	22	40
CHR-7	72	34	47
CHR-8	53	27	51
CHR-9	55	22	40
CHR-10	55	23	42
Total	670	297	44

Table 2: Molecular markers score for favorable alleles during introgression of carotenoid pathway genes into in house elite lines.

Gene	Class	Maize lines	3001 (1)	3003 (2)	1003 (3)	3866 (4)	3872 (5)	5732 (6)	3892 (7)	3896 (8)	Favourable alleles
ε-LCY	A	5′TE	2	2	2	2	3	2	0	2	2
	B	3′Indel	0	0	0	0	0	8	0	0	8
β-HYD 3	C	5′TE	1	1	1	1	1	2	1	1	2
	D	3′TE	2	2	2	2	2	1	2	2	1
	E	DEL4	0	0	0	0	0	12	0	0	12

The maize lines were further screened for the β-carotene favorable genes. Initial screening was carried out using SSR markers and the polymorphism was observed. Among all the chromosomes, CHR-5 showed high percentage of polymorphism with 44%. In the current study, ε-LCY gene and β-HYD 3 were used for screening the best carotenoid maize elite line. Research reports showed that the polymorphism at the ε-LCY loci will increase the β-carotene in the maize (Alhassan *et al.,* 2016). Results obtained in the present study showed that the 5732-cultivar showed high similarity for both ε-LCY gene and β-HYD 3 genes which shows its ability to secrete high β- carotene content. The maize line 5732 was further used for the inbreeding techniques.

CONCLUSION

The present research was carried out to screen best β-carotene maize line. The results obtained were encouraging and the 5732 was screened as the best β- carotene secreting maize line which was characterized by using biochemical and molecular analysis. The 5732 maize line is promising and can be used for the inbreeding.

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Conflict of interest

Authors have no conflict of interests.

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