



Identification of high β -Carotene lines of maize in backcross (BC_2F_1) population by genotyping *crtRB1* Allele specific marker

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ABSTRACT

In order to address the global problem of vitamin A deficiency, biofortification is considered to be a cost effective and sustainable approach. The polymorphism in *crtRB1* gene is associated with β -carotene levels in maize kernels. The randomly selected 10 seeds of selected maize lines in BC_2F_1 population cobs when analyzed for β -carotene content through HPLC, showed confirmation of improvement in β -carotene content of 2.37 $\mu\text{g/g}$. The *crtRB1* 3' TE polymorphism in BC_2F_1 population of the cross revealed that the progenies showed segregation distortion (SD). The present study indicated the possibility to develop a high β -carotene inbred with genetic identity closer to that of the recurrent parent.

Key words: Biofortification, Maize, Vitamin A, *Zea mays*, β -carotene.

INTRODUCTION

Vitamin A is an essential nutrient needed by humans. Among the nutritional quality improvement programme, alleviating the vitamin A deficiency is of global health concern. Vitamin A deficiency (VAD) causes about 70 per cent of childhood deaths worldwide and blindness in 0.25 to 0.5 million children every year (Vignesh *et al.*, 2012). Such a deficiency is associated with susceptibility to infection, night blindness, rough and scaly skin, diminished teeth and bone development. Humans are unable to synthesize their own vitamin A, which need to be provided through diet as pro-vitamin A carotenoids, including β -carotene, β -carotene (βC) and β -cryptoxanthin (βCX) of plant origin, which can be converted to vitamin A through animal metabolism (Yan *et al.*, 2010).

One of the methods to address the vitamin A deficiency worldwide is to breed food crops for increased β -carotene content, termed pro-vitamin A biofortification. Pfeiffer and McClafferty also expressed that "biofortification" is the process of development of micronutrient-enriched staple plant foods through plant breeding which holds significant promise for sustainable food-based solutions to the hidden hunger.

One of the target food crops for provitamin-A biofortification is maize (*Zea mays* L.). Maize is considered to be an ancient allo-tetraploid (Gaut and Doebly, 1997) and its genome is ten times bigger than the rice genome, owing to the expansion of families of transposable elements, particularly retro-transposons (Sanmiguel and Bennetzen, 1998). The type and number of retro-transposons vary

between inbred lines and some genes are not always shared by all inbred lines (Brunner *et al.*, 2005). The approach of biofortification in maize being taken by Harvest Plus, a program established by the Consultative Group on International Agriculture Research (CGIAR), focuses on the improvement of pro-vitamin A, iron and zinc concentrations in grain through both conventional plant breeding and modern biotechnology (Pfeiffer and McClafferty, 2007).

The four predominant carotenoid compounds in maize grain are β -carotene, β -cryptoxanthin, zeaxanthin and lutein (Figure 1) (Harjes *et al.*, 2008). However only β -carotene has the highest pro-vitamin A activity, and moreover it is present only in a relatively low concentration in maize kernels. Yellow kernel maize is thought to be associated with high levels of β -carotene, a source of vitamin A (Buckner *et al.*, 1990). Yan *et al.* (2010) confirmed that *Zea mays crtRB1* gene [encodes for β -carotene hydroxylase 1] which converts β -carotene (which has 100% provitamin A activity) to β -cryptoxanthin (which has 50% provitamin A activity) and Zeaxanthin (which has 0% provitamin A activity) by hydroxylation genes (Figure 2) (Yan *et al.*, 2010; Vignesh *et al.*, 2012 and Babu *et al.*, 2012) is an important gene associated with β -carotene metabolism in maize kernels and identified that three different polymorphisms in *crtRB1* gene viz., 5'TE, InDel4 and 3'TE, are responsible for significant variation of β -carotene concentration in kernels. Owing to the polymorphism of 3'TE (that spans the 6th exon and the 3' UTR) (Yan *et al.*, 2010 and Vignesh *et al.*, 2012), the *crtRB1* gene exists in three allelic states; 3'TE allele 1 (without insertion), 3'TE allele 2 (with 325 bp insertion),

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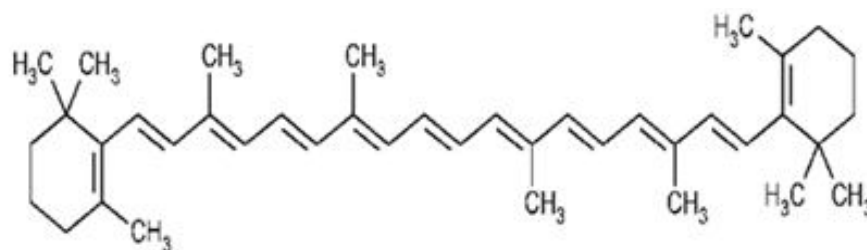


Fig 1: Structure of β -carotene

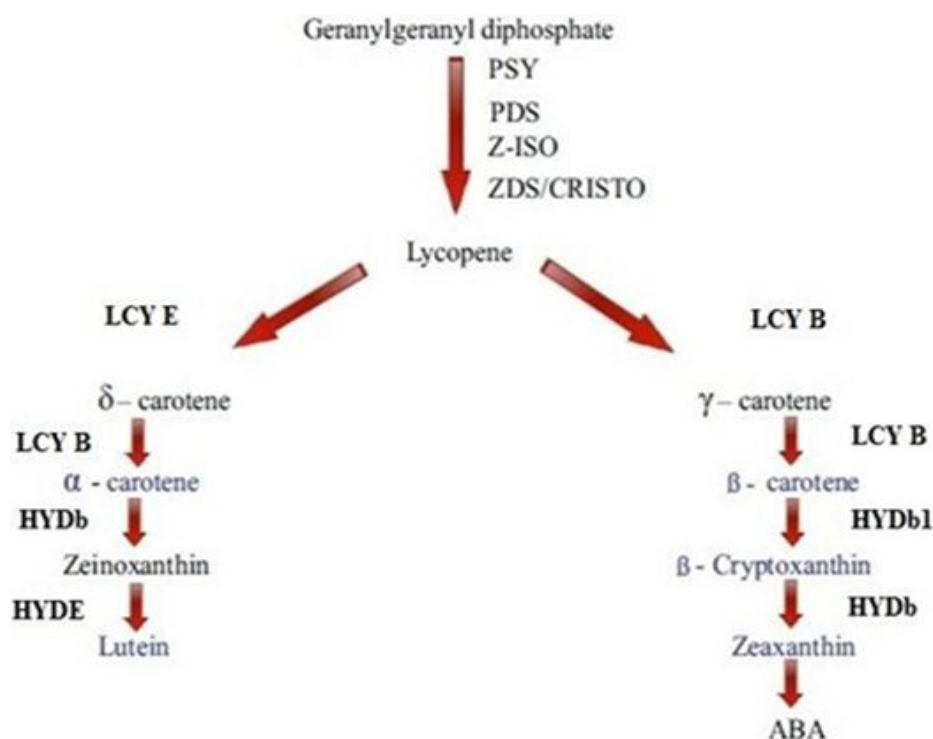


Fig 2: Carotenoid biosynthetic pathway in plants

and 3'TE allele 3 (with 1250bp insertion). The 3'TE allele hierarchy according to kernel β -carotene content is 1>3>2, which means allele 1 is more favourable than the alleles 2 and 3 for higher β -carotene accumulation in kernels and hence allele 1 is termed as favourable allele and alleles 2 and 3 are termed as unfavourable alleles (Figure 3) (Yan *et al.*, 2010).

In Marker-Assisted Back Crossing (MABC), crossing is done to transfer (introgress) favourable traits from a donor plant into an elite genotype (recurrent parent). Subsequently, backcrossing is done to recover the recurrent parent genome. In traditional backcross breeding, the recovery of the recurrent parent genotype requires more than six generations, while this may be reduced to only three generations in Marker-Assisted Back Crossing (MABC) (Tanksley, 1989). Markers can be used in the context of Marker-Assisted Back Crossing (MABC) to either control the target gene (foreground selection) or to accelerate the recovery of the recurrent parent genotype (background

selection). Babu *et al.* (2012) validated the effects of *crtRB1*-3'TE polymorphism in 26 diverse tropical genetic backgrounds and concluded that *crtRB1*-3'TE favorable allele had large and significant effect (a two to tenfold effect) on enhancing β -carotene and total provitamin A content. The present study was taken up with the following objectives

1. Phenotyping of β -carotene content in BC₂F₁ progenies through HPLC.
2. Identification of lines having favourable *crtRB1* allele in BC₂F₁ population.
3. Generation of β -carotene rich BC₃F₁ progeny by backcrossing BC₂F₁ population with recurrent parent UMI1200.
4. Generation of BC₂F₂ progenies by selfing selected BC₂F₁ population.

MATERIALS AND METHODS

As per the continuation of previous work the BC₂F₁ seeds were collected from the ongoing GOI- DBT project

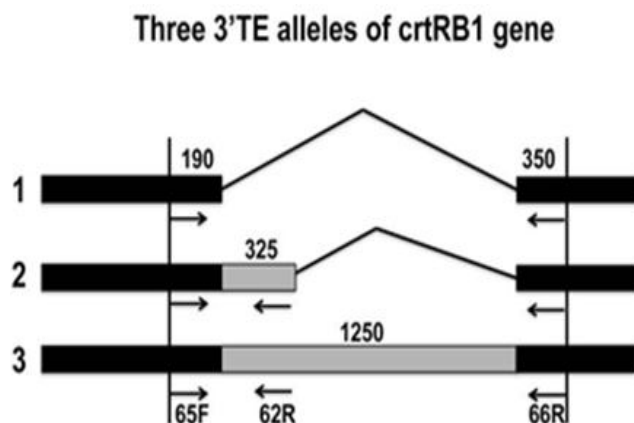


Fig 3: Genetic variation at crtRB1 3' TE

(Modified from Yan *et al.*, 2010). TRhe genetic variation due to insertions at "3TE" (a polymorphic site on the crtRB1 gene results in an amplicon of size 543bp. Allele 2 has amplicon of size 296 + 875bp. Allele 3 has a 1250bp insertion and a PCR of this allele with the indicate primer sets results in an amplicon of size 296+1221+1800. (However, the largest amplicon (1800 bp) amplified by the primers 65F and 66R was usually weak or not amplified.) Of the three alleles of 3'TE, allele 1 is known as a favorable allele, for it is associated with the enhancement of β -carotene concentration in the maize grain.

"Marker assisted introgression of Lycopodium gene for enhanced ProA in maize". In the present study BC₂F₁ progenies were analyzed for β -carotene content by HPLC. The selected progenies which were having high β -carotene were raised along with recurrent parents in *rabi*, 2013 and field studies were carried out at Eastern block at Tamil Nadu Agricultural University (TNAU), Coimbatore (11° 1' 6" N, 76° 58' 21" E). Genotyping of 3'TE polymorphism in crtRB1 gene of high β -carotene maize lines of BC₂F₁ population were done at the Genomics and Proteomics laboratory at Department of Plant Molecular Biology and Bioinformatics, Tamil Nadu Agricultural University, Coimbatore, during 2013-2014.

Evaluation of β -carotene content: The randomly selected 10 seeds of each selected BC₂F₁ cob were analyzed for β -carotene content through HPLC for confirmation of improvement in β -carotene content.

Extraction of carotenoid and quantification of β -carotene content: 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 (Weber 1987). The carotenoid extract prepared as per the Harvest plus method. Quantification of the β -carotene was carried out using a HPLC System (Shimadzu HPLC Analytical C18G 120A column 250 x 4.6 mm). Samples were eluted through YMC carotenoid C18 column and detected with a photodiode array detector. The mobile phase consisted of Acetonitrile: Methanol: Ethyl acetate (80:10:10) at high pressure through the column, and the flow rate was 1 ml min⁻¹. Standards for β -carotene was purchased from M/s. Sigma Aldrich India, reconstituted in

acetone to five different concentrations (0.1 μ g/g; 1 μ g/g; 10 μ g/g; 50 μ g/g; 100 μ g/g) (10) and were used to make the standard curve for β -carotene. 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 453 nm. The chromatogram was compared with that of the standards in order to calculate the amount of β -carotene in the samples. The concentration of β -carotene content was calculated using the following formula:

$$Cx (\mu\text{g/g}) = \frac{Ax \cdot Cs \cdot \text{Total Volume of the extract (ml)}}{\text{Sample weight in mg} \times As}$$

Where,

Cx = concentration of β -carotene in sample;

Ax = peak area of sample;

Cs = concentration of β -carotene in standard and

As = peak area of the standard

Identification of lines having favourable crtRB1 allele in BC₂F₁ progenies: BC₂F₁ progenies confirmed for high β -carotene were raised along with recurrent parents separately. All the cultural operations were carried out as per the recommendations of crop production guide of Tamil Nadu. The population so produced were screened with the foreground marker viz., crtRB1 3'TE gene based marker for confirmation of presence of favourable allele 1 (543 bp). The 3'TE polymorphism of crtRB1 gene that 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.

Screening for crtRB1 3'TE polymorphism

Seedlings were grown in the field for 3 weeks after which fresh leaf tissue of 5-6 seedlings of each inbred - 80°C for total genomic DNA extraction using CTAB method (Dellaporta SLJ. Wood and JB Hicks, 1983). The quality and quantity of DNA was determined by spectrophotometer absorbance at 260 nm. Polymerase chain reaction was performed using crtRB1 3'TE gene-specific primers (65F: ACACCACATGGACAAGTTCG, 62R: ACACTCTGGCCCATGAACAC 66R: ACAGCAATACAGGGGACCAG) and PCR amplification was carried out as follows I Hold: 95°C for 5 minutes, II Hold: Denaturation: 94°C for 1 minute, Annealing: 62°C upto 54°C for 30 seconds (19 cycles, reducing 0.5°C per cycle) and extension: 72°C for 1 minute, III Hold: Denaturation: 95°C for 1 minute, Annealing: 54°C for 1 minute and Extension: 72°C for 1 minute (19 cycles), IV Hold: Final Extension, 72°C for 10 minutes. Amplified fragments were separated using 1.5% agarose gel based electrophoresis and were scored for the presence of favorable alleles.

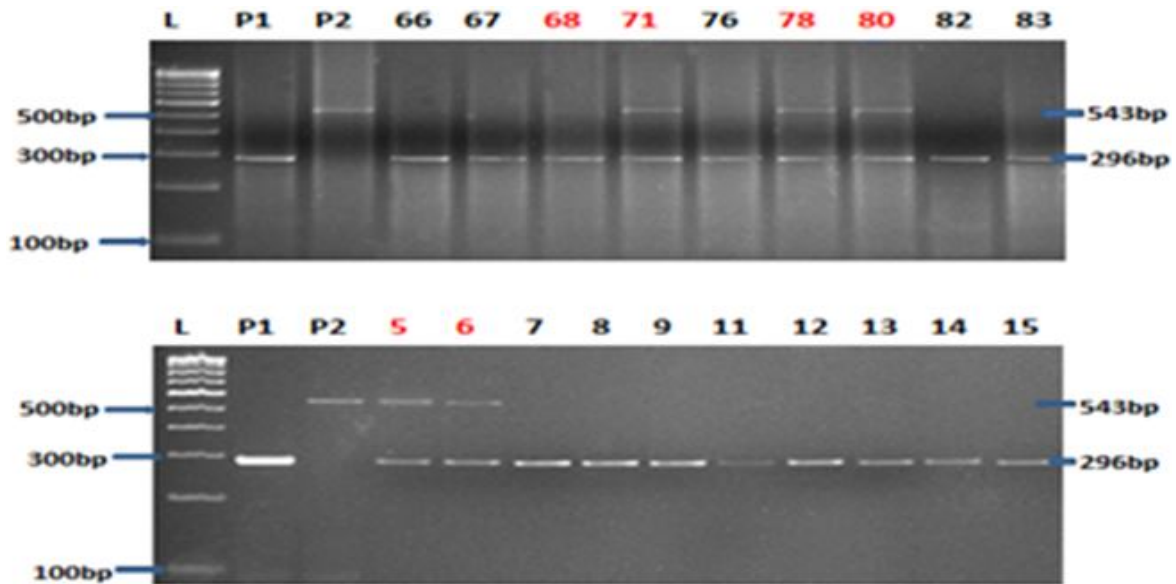


Fig 4: Screening of foreground marker in BC₂F₁ population

(L denotes marker Lane. P1& P2 denotes parents UMI1200 and HP46715 respectively)

Development of BC₃F₁ and BC₂F₂ generation: The BC₂F₁ progenies which were confirmed for high β -carotene content using HPLC and molecular markers were forwarded to develop further generation. The selected BC₂F₁ population was back crossed with the recurrent parent UMI 1200 to produce BC₃F₁ generation. Crossing and Selfing was carried out adopting tassel bag method in which ear shoot of maize emerging from leaf sheath was bagged with butter paper cover to protect the silk from foreign pollen contamination through wind pollination for two days before silk emergence. The brown paper cover was placed over the tassel of the male parent on the previous day of the pollination to collect pollen. The pollen collected in the tassel bag was dusted over the emerged silk after removing the butter paper cover and replaced immediately to avoid contamination. A brown paper cover was tightly fastened over the pollinated ear for protection. Manual pollination was practiced between 9 to 11 A.M.

RESULTS AND DISCUSSION

Estimation of β -carotene content ($\mu\text{g/g}$) BC₂F₁ progenies:

The results revealed that β -carotene content of BC₂F₁ was found to be 2.37 $\mu\text{g/g}$ and the progeny was forwarded to develop BC₂F₁ population.

Foreground marker screening in BC₂F₁ [pedigree: (UMI 1200 $\times$ HP467-15)-C15-C22] population: Among 100 progenies, 26 progenies showed both parent's alleles 'AB' (Heterozygotes with amplicon size of 296+543 bp) while 74 progenies showed 'A' alleles (Amplicon size of 296 bp) (Figure 4). The Chi-square test for goodness of fit was calculated for the allele frequency revealed that g^2 (df=1) at 0.5 per cent level was 3.84 and at 1 per cent level it was 6.635. The calculated value was more than table value. All the progenies did not showed the expected segregating ratio (1:1) with respect to the *crtRB1* gene specific marker. In BC₂F₁ population, segregation disorder (SD) was skewed toward the unfavourable homozygous allele and the favourable allele was under-represented in the BC₂F₁ population (Table 1)

Development of BC₃F₁ progenies: The individuals possessing the *crtRB1* loci in heterozygous condition (AB) with amplicon size of (296+543bp) were used as female parent for further back crossing with male parent (UMI 1200) to produce BC₃F₁ generation [pedigree:(UMI 1200 $\times$ HP 467-15)-C15-C22-C10].

Improvement in β -carotene content by introgression of favourable *crtRB1* allele from exotic donor line

Table 1: Chi-square goodness of fit for BC₂F₁ segregating population [pedigree:(UMI 1200 $\times$ HP467-15)-C15-C22]

Population	Marker	Total No. of plants	Observed values			χ^2
			Heterozygous (AB)	Homozygous (AA)	Homozygous (BB)	
(UMI 1200 $\times$ HP467-15)-C15-C22	<i>crtRB1</i>	100	26	74	-	12.224

The table value of χ^2 (df =1) at 5% level is 3.84 and 1% level it is 6.64 The calculated value is more than calculated value. There is deviation between expected ratio and observed ratio.

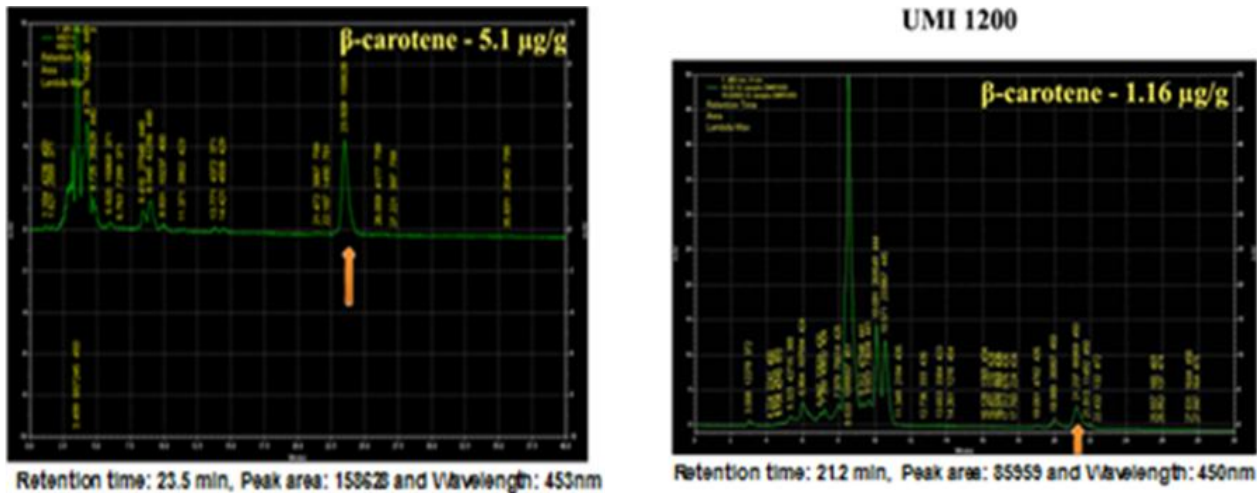


Fig 5: HPLC Chromatogram for beta carotene content of Donor (HP467-15) and Recurrent (UMI1200) parents

(HP467-15) into popular inbred (UMI 1200) through marker assisted: backcrossing.

Both Recurrent (UMI 1200) and Donor (HP467-15) parents were analyzed for β -carotene content through HPLC (Figure 5) and the results showed that UMI 1200 (Recurrent parent) has β -carotene content of 1.16 $\mu\text{g/g}$ and HP467-15 (Donor parent) has β -carotene content of 5.1 $\mu\text{g/g}$.

The randomly selected 10 seeds in each of the selected BC_2F_1 cob were analyzed for β -carotene content through HPLC and the results showed confirmation of improvement in β -carotene content of 2.37 $\mu\text{g/g}$ for BC_2F_1 progeny (Figure 6) [pedigree: (UMI1200 $\times$ HP467-15)-C15-C22]. (Table 2) (Plate 1).

In traditional backcross breeding, the reconstruction of the recurrent parent genotype requires more than six generations, while this may be reduced to only three

Table 2: HPLC analysis of β -carotene content of parents (UMI 1200 and HP467-15) and selected BC_2F_1 progeny.

Parents/progenies	Pedigree	β -carotene($\mu\text{g/g}$)
Recurrent	UMI1200	1.16
Donor	HP467-15	5.1
BC_2F_1	(UMI1200 $\times$ HP467-15) -C15-C22	2.37

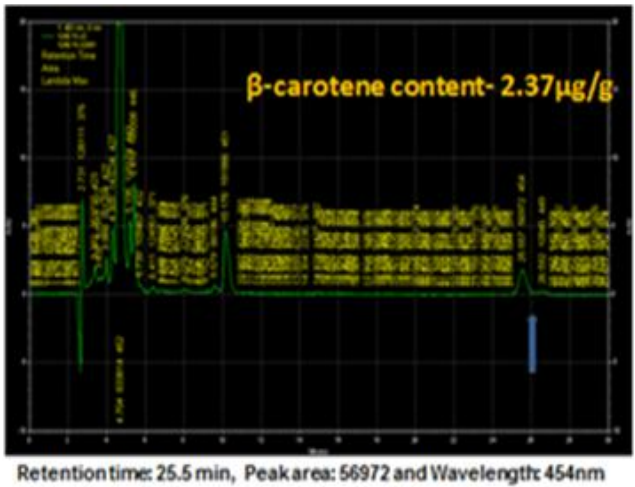


Fig 6: HPLC Chromatogram for beta carotene content of BC_2F_1 progeny



Plate 1: Variability in crop characters

generations in Marker-Assisted Back Crossing (MABC) (Tanksley *et al.* 1989). In addition, it is said to be cost effective and time-saving (Eathington *et al.*, 1997). Maize breeders try to combine the nutritional quality traits with yield to produce nutritive inbreds. Selection of favorable gene alleles with inexpensive molecular markers will enable breeders to produce more effectively maize grain with higher provitamin A levels.

In the present study BC₂F₁ progenies were analyzed for β -carotene content by HPLC and the results showed confirmation of improvement in β -carotene content of 2.37

$\mu\text{g/g}$ (Selvi., 2014). The analysis of 3'TE allele polymorphism of *crtRB1* gene in BC₂F₁ population revealed that the progenies showed segregation distortion (SD) [i.e., deviation from the expected segregating ratio, 1:1 for BC₂F₁ populations], indicating that the favourable allele is under-represented in the population. The present results were in confirmation with the earlier reports. Lu *et al.* (2002) opined that the observation of segregation disorder (SD) for *LcyE* in all eight and for *crtRB1* in five of the eight digenic F₂ populations was consistent with frequent observation of SD in maize and presence of many segregation distortion regions (SDRs) throughout the maize genome.

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